The effect of nitrogen status and carbon remobilisation on nitrate assimilation and associated cytokinin signalling in shoots and roots of *Lolium perenne*

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ABSTRACT

The efficiency of nitrogen (N) assimilation is a critical component of N fertiliser use by plants, regrowth following defoliation and forage production in *Lolium perenne* L., an important pasture species worldwide. The aim of this study was to gain insight into the effect of modifying the internal carbon (C)/N balance on the efficiency of nitrate (NO_3^-) assimilation and to determine the relevance of the cytokinins to this process.

The physiological and biochemical responses to perturbation of the internal C/N balance by N treatment to plants grown for 10 weeks in unfertilised soil, defoliation, and exposure to contrasting day length and N supply, were characterised over a seven-day period in the leaf, sheath and root of perennial ryegrass. A spatiotemporal analysis of NO_3^- use efficiency (NUE) was described in terms of growth response, remobilisation of the water-soluble carbohydrates (WSCs) across their degrees of polymerisation, amino acid profile, together with components of cytokinin signal transduction. The expression of the cytokinin response regulator *LpRR* genes was monitored by RT-qPCR.

The initial response to high levels (5 mM) of KNO_3 addition to N-impoverished plants was characterised by a rapid remobilisation of carbon stores from the low-molecular weight WSC, along with an increase in N content and assimilation into free amino acids. Subsequently, the shoot became the main source of C through remobilisation of a large pool of high-molecular weight WSC. Variations in the cytokinin content and *LpRR* gene expression suggest long-distance root/shoot signalling within a day of KNO_3 treatment, and ongoing input from cytokinin over seven days. The expectation that perennial ryegrass possesses a cytokinin-mediated N-signalling/relay system similar to that characterised in model plant species *Arabidopsis thaliana* was confirmed. The presence of high levels of *cis*-zeatin-type cytokinins was considered in the context of hormonal homeostasis under the stress of steady-state N-deficiency.

Plants, manipulated to have contrasting internal C/N balance, were obtained by exposure to either continuous light or short days (8 h light: 16 h dark), and watered with modified N-free Hoagland medium containing either high (5 mM) or low (50 µM) NO_3^- as sole N source. An increase in C demand was experimentally induced by a defoliation of perennial ryegrass

plants. Leaf regrowth following defoliation involved remobilisation of the low- and high-molecular weight WSCs. The extent of the remobilisation and the partitioning of the WSC following defoliation were dependant on the initial WSC levels and on the C and N availability to the plant. Cytokinin levels varied in the sheath and root as early as 8 h following defoliation and preceded an overall increase in amino acids at 24 h. Within 168 h after cutting, a negative feedback brought the amino acid response back towards pre-defoliation levels, and was under control of the C/N ratio. Therefore, it is suggested that WSC remobilisation in the leaf is coordinated with N availability to the root via systemic cytokinin signal leading to efficient N assimilation in the leaf and in the sheath tissues and to early leaf regrowth following defoliation.

The effect of exogenous application of a cytokinin-like compound, CPPU, was tested as a potential signal "priming" the remobilisation of WSCs reserves required for NO_3^- assimilation. Foliar spray of CPPU did not increase the physiological response of perennial ryegrass plants to N treatment and defoliation, as measured by biomass of regrown leaf material, tiller and leaf number, leaf elongation rate, maximum photosynthetic rate or stomatal conductance. Hydroponic treatment with a cytokinin receptor antagonist, referred to as PI-55, influenced the activation of the cytokinin signalling pathway but without physiological consequences.

Based on the results obtained in the present study, the development of a cytokinin-based N fertiliser would not be expected to improve the NUE of perennial ryegrass. However, based on the effect of N treatment and defoliation on the remobilisation of the WSCs, a recommendation is made that a grazing event and N fertiliser application should be separated in time by several days to allow a recovery of some of the WSC reserves.

ABBREVIATIONS

°C	degrees Celsius
6-BAP	6-benzylaminopurine
ACN	acetonitrile
AHK	*Arabidopsis* histidine kinase
AHP	*Arabidopsis* histidine phosphotransfer protein
Ala	alanine
ALD	aldolase
A_{max}	maximum photosynthetic capacity
AMT	ammonium transporter
ANOVA	analysis of variance
AQC	δ-aminoquinolyl-N-hydroxysuccinimidyl carbamate
Arg	arginine
ARR	*Arabidopsis* response regulator
AS	asparagine synthetase
Asn	asparagine
Asp	aspartic acid
ASP	aspartate aminotransferase
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
C	carbon
cDNA	complementary deoxyribonucleic acid
CF	correction factor
CKX	cytokinin oxidase/dehydrogenase
CPPU	*N*-(2-chloro-4-pyridyl)-*N*′phenylurea
CRE	cytokinin response
Ct	threshold cycle
CYP735A2	cytokinin *trans*-hydroxylase
Cys	cysteine
*c*Z	*cis*-zeatin
*c*Z9G	*c*Z-9-glucoside

*c*ZOG	*c*Z *O*-glucoside
*c*ZR	*c*Z riboside
*c*ZROG	*c*Z riboside *O*-glucoside
*c*ZRMP	*c*Z ribosyl monophosphate
d	day
DHZ	dihydro-zeatin
DHZ7G	DHZ-7-glucoside
DHZ9G	DHZ-9-glucoside
DHZOG	DHZ *O*-glucoside
DHZR	DHZ riboside
DHZRMP	DHZ ribosyl monophosphate
DHZROG	DHZ riboside *O*-glucoside
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxy-nucleotide-triphosphate
DM	dry matter
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DP	degrees of polymerization
DW	dry weight
E	east
EDTA	ethylene diamine tetra-acetic acid
EF	elongation factor 1 alpha
ENO	enolase
EST	expressed sequence tag
FA	formaldehyde
FAD	flavin adenine dinucleotide
g	gravitational acceleration
g_s	stomatal conductance
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	gas chromatography
GDH	glutamate dehydrogenase
Gln	glutamine
GLN	glutamine synthetase

GLT	NADH-glutamate synthase
Glu	glutamic acid
Gly	glycine
GOGAT	glutamine-2-oxoglutarate aminotransferase
GS	glutamine synthetase
h	hours
HATS	high affinity transport system
HCl	hydrochloric acid
His	histidine
HMW	high-molecular weight
HPLC	high-performance liquid chromatography
Hz	hertz
Ile	isoleucine
INV	invertase
iP	isopentenyladenine
IPA	isopropanol
iP7G	iP-7-glucoside
iP9G	iP-9-glucoside
iPR	iP riboside
iPRMP	iP riboside monophosphate
IPT	isopentenyl transferase
IRGA	infrared gas analyser
IRMS	isotope ratio mass spectrometer
kb	kilobase
kg	kilogram
kV	kilovolt
l	litre
LATS	low affinity transport system
LC	liquid chromatography
Leu	leucine
LMW	low-molecular weight
Lys	lysine
m	meter
M	molar

Met	methionine
mg	milligram
MgCl	magnesium chloride
min	minute
MRM	multiple reaction-monitroring
mRNA	messenger ribonucleic acid
ms	millisecond
MS	mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
N	nitrogen
NaOH	sodium hydroxide
NCBI	national centre for biotechnology information
NiR	nitrite reductase
NJ	neighbor-joining
NR	nitrate reductase
NRT	nitrate transporter
NUE	N use efficiency
NUpE	N uptake efficiency
NUtE	N utilisation efficiency
NZ	New Zealand
Orn	ornithrine
P	phosphate
PCR	polymerase chain reaction
PGAM	phosphoglycerate mutase
PGI	glucose-6-phosphate isomerase
Phe	phenylalanine
PI-55	6-(2-hydroxy-3-methylbenzylamino)purine
PK	pyruvate kinase
PPFD	photosynthetic photon flux density
ppm	part per million
Pro	proline
QTOF	quadrupole-time of flight
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolution per minute
RR	response regulator
RT	retention time
RT-qPCR	quantitative reverse transcription polymerase chain reaction
s	seconds
S	south
SAM	shoot apical meristem
SD	standard deviation
SE	standard error
Ser	serine
T	time
TCA	tricarboxylic acid
TF	transcription factor
Thr	threonine
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose-6-phosphate synthase
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
TRE	trehalase
Trp	tryptophan
Tyr	tyrosine
*t*Z	*trans*-zeatin
*t*Z7G	*t*Z-7-glucoside
*t*Z9G	*t*Z-9-glucoside
*t*ZOG	*t*Z *O*-glucoside
*t*ZR	*t*Z riboside
*t*ZRMP	*t*Z riboside monophosphate
*t*ZROG	*t*Z riboside *O*-glucoside
UPLC	ultra-performance liquid chromatography
Val	valine
v v^{-1}	volume per volume
w v^{-1}	weight per volume
WSC	water-soluble carbohydrate

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Jessica Roche (70-75%) and Dr. Jonathan Love (20%) designed the experiments, carried out the experiments and analysed the data. Qianqian Guo (5-10%) assisted with the experiments.

Jessica Roche wrote the manuscript (85%) with significant input from Prof. Paula E. Jameson and comments from all other authors (25%).

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Dr. Ondřej Novák provided the cytokinin analysis (95%) on samples prepared by Jessica Roche (5%) at the University of Canterbury.

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Name: *Roche, Love, Guo, Song, Cao, Fraser, Huege, Jones, Novák, Turnbull, Jameson*

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Name: *Roche, Turnbull, Guo, Novák, Späth, Gieseg, Jameson, Love*

Signature: *Jameson*

Date: *05.09.2016*

Chapter 1

Introduction, review of literature and rationale

Contents

1. 1 Background

Agriculture and downstream processing sectors are essential components of the New Zealand economy and account for around 8% of the gross domestic product, with dairy predominant in this sector, followed by beef, sheep, and horticulture (economic and financial overview 2016, treasury report from the New Zealand government). Agriculture of the 21st century will have to face new challenges such as sustaining food and fiber production for a growing human population and an adaptation of the agricultural practices to mitigate climate change, and not compromise environmental integrity (Takei et al. 2002). Over the past two decades, New Zealand agriculture has been characterised by a conversion of sheep to dairy farming, and by an intensification of fertiliser inputs (Garnett et al. 2009, Foote et al. 2015). Pasture and arable land combined cover 53% of New Zealand total land area. This consists predominantly of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) (Van Wijk et al. 1993, Daigneault et al. 2008, Dawar et al. 2010, Dawar et al. 2012).

Perennial ryegrass is a pasture plant which has a central role in the pastoral systems throughout the world due to its agronomical advantages (Humphreys et al. 2010). It has become the most widely sown perennial forage grass in temperate regions of the USA, China, Japan, UK, Australia, New Zealand, South Africa, and South America (Wilkins and Humphreys 2003). Perennial ryegrass agronomical advantages include ease of establishment, good tolerance to grazing, high digestibility, and adequate seed production with a more extended season of active growth than many other species (Wilkins 1991). Grass species such as *L. perenne* are characterised by vegetative meristems located at the shoot base, which offers a physiological protection for regrowth and for plant survival following grazing/defoliation (Langer 1979). Defoliation causes an immediate decrease in root growth, photosynthesis, respiration, and nutrient uptake (Clement et al. 1978, Parsons et al. 1983, Jarvis and Macduff 1989). Improvement of perennial ryegrass nitrogen use efficiency on-farm requires an extended understanding of the mechanisms integrating carbon- and nitrogen-metabolism following fertiliser application during the grazing cycle.

The mineral nutrient required in the greatest amount by plants is nitrogen (N). Nitrogen is an essential constituent of amino acids, proteins, nucleic acids, chlorophyll *a*, and many secondary metabolites. Thus, N has a central role in synthesis of cell materials and plant

tissues and N corresponds to 1.5 - 2% of plant dry-matter (Marschner 1995). In many of the world's agricultural areas, N was identified as the most limiting nutrient for plant growth and crop productivity (Morrison 1958). Intensive forage production relies on irrigation and on N fertiliser input supplied mainly as inorganic nitrate (NO_3^-), ammonium (NH_4^+), and urea (Dawar et al. 2011, Dechorgnat et al. 2011). In 2014, 102 megatons of N fertiliser were applied globally (FAO, 2014). The industrial production of N fertilisers through the chemical Haber-Bosch process is estimated to be responsible for sustaining roughly 40% of the world's population and is the source for 40-60% of N found in the human body. Nearly one century after its invention, the process is still used globally for an annual production of 500 million tons of artificial fertiliser (Fryzuk 2004). Bolan et al. (2004) reported that New Zealand agricultural fields receive about 3 million tons of N per year, of which half originates from the urine and faeces of farm animals (Bolan et al. 2004). The application of N fertiliser in New Zealand has resulted in a six-fold increase in elemental N from 60 000 tonnes in 1990 to 350 000 tonnes in 2013 (Ministry for the Environment, 2013).

With only 30 to 40 % of applied N utilised by the plant, intensive use of nitrogenous fertiliser is increasingly causing negative short- and long-term effects on the environment, and on farmer economies (Masclaux-Daubresse et al. 2010). Soil denitrification, surface runoff, leaching, volatilisation, and fluxes of other gaseous emissions to the atmosphere are responsible for the majority of fertiliser loss (Carpenter et al. 1998, Raun and Johnson 1999). Indeed, NO_3^- is highly mobile and can move with percolating water out of the soil, making it unavailable for plant uptake and increasing N loads on recipient waterways. This process can lead to algal blooms and eutrophication characterised by hypoxic conditions destabilising the aquatic ecosystem and resulting in substantial loss of species diversity (Smith et al. 1999, Wang et al. 2012b, Bouwman et al. 2013). Moreover, excess NO_3^- levels in drinking water have been associated with reduced ability of the blood to carry oxygen, which causes rare cases of "blue baby syndrome" (Knobeloch et al. 2000). Due to this health risk, the world health organisation defined a water maximum contaminant level of 3 mg/l for nitrite, 50 mg/l for nitrate, and a sum of ratios of the concentration of nitrite and nitrate to their guideline value not exceeding one when nitrite and nitrate simultaneously present in drinking water (Gutierrez 2012).

Nitrogen fertilisers can stimulate the soil microbial activity to convert N fertiliser into gaseous nitrous oxide (N_2O) emissions released in the stratosphere during a process called

denitrification (Mosier et al. 1998). Nitrous oxide is a greenhouse gas considered to be 300 times more potent than CO_2 and was ranked the third most abundant greenhouse gas, behind carbon dioxide (CO_2) and methane (CH_4) (Seinfeld 1986, Montzka et al. 2011, McAllister et al. 2012). An increase in ambient temperatures, humidity, and well-aerated soils are associated with greater emissions. Atmospheric pollution associated with N fertiliser by-products plays a potential role in global warming (Vitousek and Howarth 1991). Finally, the excessive use of NH_4^+-based N fertilisers in crop production has been shown to acidify soils, which results in a decrease of the cation exchange capacity, base saturation, and exchange of calcium and manganese (Barak et al. 1997). The most detrimental effects from soil acidity are due to aluminium and manganese toxicities which increase as the soil pH drops below 5 (Wright 1989).

Key strategies to consider for a more sustainable agriculture are the targeting of N for optimal acquisition by plants, the selection of cultivars that use N more efficiently, and the development of a better mechanistic understanding of N use regulation (Hirel et al. 2007).

1. 2 Nitrogen use efficiency

1.2.1 Nitrogen availability and dry matter partitioning

Nitrogen use efficiency (NUE) is an important parameter commonly used to assess relative efficiency of fertiliser input to farmland (see Andrews and Lea (2013) for review on improving crop NUE). Several definitions can be found in the literature. In this study, NUE refers to the plant biomass produced per unit of applied N. Nitrogen use includes the steps of N uptake, translocation, assimilation, and remobilisation (Xu et al. 2012). Nitrogen use efficiency can be identified as the sum of the efficiency of N uptake (NUpE) from the exogenous environment by plant roots and of N utilisation efficiency (NUtE), which converts N taken up into an ultimate plant growth response (Good et al. 2004, Hirel et al. 2007, Masclaux-Daubresse et al. 2010).

Nitrogen use efficiency depends on the physiology of the plant as well as on N form and availability to the plant (Andrews et al. 2013). Agronomic practices, such as grazing cycles, crop rotation, tillage, the irrigation system, and methods of N fertiliser application, can greatly influence the N resources available for uptake (Hatfield et al. 2001). In addition,

microbial and fungal interactions as well as abiotic factors create a highly heterogeneous soil environment with dynamic variations in NO_3^- and NH_4^+ concentrations (Adesemoye and Kloepper 2009, Xu et al. 2012). Nitrogen, especially the NO_3^- form, controls many aspects of plant metabolism and development (Krouk et al. 2010a). Plants under N-limiting environment increase the allocation of resource towards the development of the tissue responsible for N acquisition (Davidson 1969, Mora Izquierdo et al. 2011). Physiological changes associated with N limitation include overall reduced growth, decreases in shoot to root biomass ratio, local proliferation of lateral roots in N-rich areas, and an increase in N uptake rate (Robinson and Rorison 1983, Remans et al. 2006, De Dorlodot et al. 2007, Desnos 2008, Puig et al. 2012). Conversely, an increase in N supply results in an increase of dry weight allocation to the shoot relative to the root across a wide range of plant species, such as *Pisum sativum*, *Phaseolus vulgaris*, and *Triticum aestivum* (Andrews et al. 1999, Andrews et al. 2001, Poorter et al. 2012).

Several theories have been proposed to explain the effects of N and NO_3^- on dry matter partitioning between root and shoot. Under conditions of N deficiency, Brouwer (1963) hypothesised that N taken up from the soil is directly used for root growth, and a deficit of N amount translocated from root to shoot results in a decreased root to shoot ratio (Brouwer 1963). Thornley (1972) proposed that the factors which determine shoot to root ratio are the supply of photosynthetic C by the shoot and the supply of N taken up from the external environment by the root (Thornley 1972). It was suggested that root growth is limited by supply of C from the photosynthetic leaves, and that leaf growth is limited by supply of water and nutrients from the roots (Thornley 1972). However, this model fails to explain a decrease in shoot to root ratio under N deficiency observed for plant species with the main site of NO_3^- assimilation in the shoot regardless of the external NO_3^- supply (Andrews et al. 2001). Work with mutants and transgenic plants of tobacco with decreased expression of NO_3^- reductase showed a positive correlation between accumulation of NO_3^- in the shoot and increased shoot to root ratio (Scheible et al. 1997). It was proposed that an accumulation of NO_3^- in the shoot could acts as a signal which regulates the dry matter partitioning between shoot and root in tobacco (Scheible et al. 1997). Andrews et al. (2006) observed a strong positive relationship between shoot to root ratio and content of soluble protein in the shoot. It was proposed that the shoot protein concentration reflects the availability of N for shoot growth (Andrews et al. 2006). In Andrews et al. (2007) model, an increase in N supply results in an increase of shoot to root ratio due to a local use of photosynthate and smaller proportion of C translocated from

shoot to root for root growth (Andrews et al. 2001, Andrews et al. 2006, Andrews et al. 2007).

1.2.2 Nitrogen uptake and transport

Plants have evolved with physiological and molecular plasticity to provide them with a range of capacities, affinities, and regulation of N-uptake transporters, which allows an efficient use of resources (Lea and Azevedo 2006). Both mineral N (NO_3^- and NH_4^+) and organic N forms (amino acids) can be taken up from the environment with the inorganic form generally present at greater availability and considered to be the preferred N source (Harrison et al. 2007, Näsholm et al. 2009). In addition, foliar absorption of urea, NO_3^- and NH_4^+ is possible, although at a lower rate than root absorption (Bowman and Paul 1992). Nitrate taken up from the soil can be reduced to NH_4^+ and subsequently assimilated, stored in the vacuole or transported from cell to cell for xylem loading.

Active N acquisition across the plasma membrane of epidermal and cortical root cells involves high affinity transporter systems (HATS) and low affinity transporter systems (LATS) controlled by plant demand, and by NO_3^- and NH_4^+ availability to the plant (Glass et al. 2002). Excessive NH_4^+ ions in the cell are rapidly stored in the vacuole to avoid the formation of toxic amides (Britto et al. 2001, Etxeberria et al. 2012). In *Arabidopsis thaliana*, HATS are considered to act at the concentration ranges of 10 - 250 µM NO_3^-, and at a concentration greater than 250 µM NO_3^- for the LATS (Forde 2000, Okamoto et al. 2003). Additionally, constitutive NO_3^- transport systems operate regardless of N concentration, whereas inducible NO_3^- transport systems and NO_3^- transporter genes (*NRT*) are regulated as a consequence of the detection of NO_3^- availability by the roots (Glass et al. 1992).

Nitrate uptake, allocation, and storage are under the control of four gene families: nitrate transporter 1 (*NRT1*), *NRT2*, chloride channel (*CLC*), and slow anion channel associated 1 homolog 3 (*CLAC1/SLAH*) (Wang et al. 2012b). The recent discoveries that *NRT1* family also includes transporters of amino acids, peptides and hormones resulted in new terminology as *NRT1/PTR* family (*NPF*) (Forde 2000, Léran et al. 2014, Chiba et al. 2015). Table 1.1 summarise the functions and regulations of *A. thaliana* NO_3^- transporters.

Table 1.1. Summary of physiological functions and regulations of *A. thaliana* NO_3^- transporters. Abbreviations: CLC, chloride channel; HATS, high affinity transport system; LATS, low affinity transport system; NAXT, nitrate excretion transporter; NRT, nitrate transporter. Modified from Wang et al. (2012b).

Gene family member	Function	Nitrate response	Other regulations
NRT1.1	Nitrate sensing HATS and LATS	Induction	Nitrite and high pH repression. Auxin, light, sugar and nitrite induction
NRT1.2	LATS	Constitutive	Not known
NRT1.3	Not known	Induction	Light induction
NRT1.4	Leaf nitrate homeostasis	Constitutive	Not known
NRT1.5	Root xylem loading	Induction	High pH and potassium limitation repression. Sugar induction
NRT1.6	Nitrate transport to embryos	Not known	Not known
NRT1.7	Nitrate remobilisation from old to young leaves	Not known	Sucrose induction
NRT1.8	Xylem unloading	Induction	Cadmium induction
NRT1.9	Nitrate loading into root phloem	Constitutive	Not known
NAXT1	Root nitrate efflux	Not known	Acidic pH induced
NRT2.1	HATS	Induction	Ammonium and glutamine repression. Light and sugar induction
NRT2.2	HATS	Induction	Not known
NRT2.4	HATS	Repression	Ammonium repression. Light induction
NRT2.7	Nitrate storage in mature embryos	Constitutive	Not known
CLCa	Nitrate accumulation in vacuoles	Induction	Not known
CLCb	Nitrate accumulation in vacuoles	Not known	Not known

Extended characterisation of *NRT* families identified that *NRT1* and *NRT2* are both able to transport NO_3^- together with a proton in a symport mechanism under the control of a membrane pH gradient (Miller et al. 2007). The gene family *NRT2* has been cloned and identified as a high-affinity transporter in a wide range of plant species, i.e. *A. thaliana* (Filleur and Daniel-Vedele 1999), *Hordeum vulgare* (Trueman et al. 1996), *Glycine max* (Amarasinghe et al. 1998). Formation of a complex with NO_3^- assimilation related gene family (*NAR2*) is required for *AtNRT2.1* to act as high affinity NO_3^- transporter (Yong et al. 2010).

Nitrate taken up from the soil is stored in the vacuole, transported from cell to cell for xylem loading, or assimilated into more complex molecules. The transport from root to leaves

follows the transpiration stream and ultimately distributes the N-compounds to mesophyll cells for storage or direct use (Tegeder and Rentsch 2010). The chloride channels gene family member *CLCb* mediates NO_3^-/H^+ exchange across the tonoplast of *A. thaliana* vacuoles, which results in NO_3^- accumulation in vacuoles (Von der Fecht-Bartenbach et al. 2010). Nitrogen accumulates to a greater overall level in the leaf relative to the other tissues (Moser et al. 1982). The amino acids, especially the primary N products glutamine, glutamate, asparagine and aspartate, can be transported throughout the plant **(**Fig. 1.1**)** (Schjoerring et al. 2002). In addition, NO_3^- can be excreted back to the external environment by nitrate excretion transporter (*NAXT1*) under conditions of excessive endogenous concentration of NO_3^- (Crawford and Glass 1998).

1.2.3 Assimilation and remobilisation of nitrogen into carbon skeletons

In perennial ryegrass plants, the sheath is the main site for C reserve (Prud'homme et al. 1992) and amino acid synthesis, and/or accumulation (Bigot et al. 1991). Amino acid biosynthesis occurs as a result of the assimilation of NO_3^- with C-accepting molecules (Delhon et al. 1995). Nitrate assimilation can take place both in the root and in the shoot depending on plant species and soil NO_3^- concentration (Andrews et al. 2001). In temperate cereals, root is the main site of assimilation at external NO_3^- concentrations up to 1 mM, and shoot assimilation increases as external NO_3^- concentrations increases (Andrews 1986). Consistently, NO_3^- treatment to N limited perennial ryegrass plants resulted in an initial phase of NO_3^- reduction in root during the first 12 hours followed in time by a greater NO_3^- reduction in shoot (Bowman and Paul 1988). The efficiency of NO_3^- assimilation depends on the regulation of the different enzymes involved in amino acid synthesis and their ultimate conversion into more complex N-containing molecules supporting growth. Initially, NO_3^- is reduced to nitrite (NO_2^-) by the cytoplasmic FAD-dependent NO_3^- reductase (NR) enzyme, and NO_2^- is subsequently converted to NH_4^+ by NO_2^- reductase enzyme (King et al.) located in the root plastids and shoot chloroplasts (Masclaux-Daubresse et al. 2010, King et al. 2012). Reduction of NO_3^- to NH_4^+ results in the production of OH^-. Maintain of the pH balance is possible by direct extrusion of OH^- into the soil by the root, and by neutralisation of OH^- with organic acids such as malate in the shoot and subsequent transport to the root (Peuke et al. 1996).

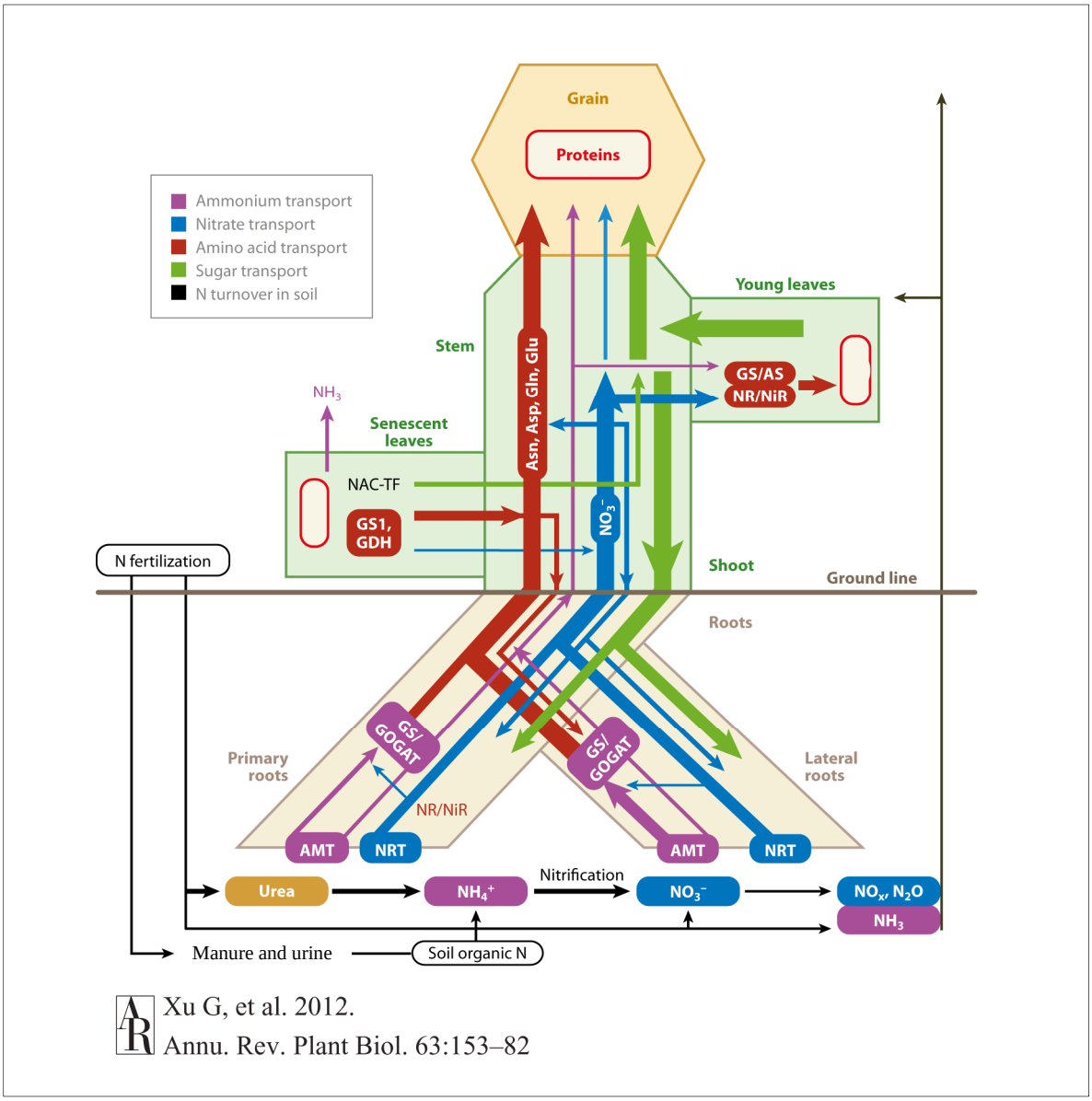


Figure 1.1. Schematic representation of nitrogen (N) uptake following fertilisation and subsequent internal transport of organic and inorganic N forms and sugars through primary and lateral roots, stem, senescent leaves, young leaves and grain. The thickness of the arrows corresponds to the relative endogenous N forms and sugar content. Abbreviations: AMT, ammonium transporter; AS, asparagine synthetase; Asn, asparagine; Asp, aspartate; GDH, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; GOGAT, glutamine-2-oxoglutarate aminotransferase; GS, glutamine synthetase; NAC-TF, transcription factors belonging to the NAC family; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate transporter (minor changes from Xu et al. (2012)).

Nitrogen assimilation into organic compounds involves the addition of NH_4^+ on a glutamate molecule by the glutamine synthetase (GS) enzyme to produce a molecule of glutamine able to react with 2-oxoglutarate by action of the glutamate synthase enzyme (GOGAT) resulting in two molecules of glutamate (Lea and Forde 1994). Two forms of the GS enzyme are present in plants: GS1 and GS2. The chloroplatic GS2 form is considered to be the one responsible for NH_4^+ assimilation, whereas the cytosol located GS1 form is involved in NH_4^+ recycling (Masclaux-Daubresse et al. 2010). In higher plants, GOGAT use either NADH or ferredoxin (Fd) as the electron carrier (Lam et al. 1996).

The NADH-GOGAT form is located in plastids of non-photosynthetic tissues and play a key role in N primary assimilation and ammonia recycling (Sechley et al. 1992), whereas Fd-GOGAT is mainly found in leaf chloroplast and seems to be implicated in light regulated processes (Suzuki et al. 1982). Ammonium or glutamine can be used by the cytosolic asparagine synthetase (AS) enzyme for an ATP-dependent production of asparagine (Lam et al. 2003).

The primary amino acids can subsequently initiate the production of other amino acids by action of aminotransferases (Forde and Lea 2007). Indeed, glutamate can be converted into arginine via a three steps N incorporation process involving an initial transamination of glutamate to give the 2-amino group of ornithine, followed by transamination to the 5-amino group of ornithine, and final N incorporation from the amide group of glutamine to the guanidine group of arginine (Miflin and Lea 1977). Nitrogen-containing compounds can be converted to more complex molecules, stored in the vacuole, or remobilised (Masclaux et al. 2006).

Remobilisation of N by ammonia recycling allows a rapid increase in endogenous N availability. Assimilation of recycled N takes place throughout the plant development, especially during leaf senescence (Diaz et al. 2008), and grain filling (Salon et al. 2001). Recycling of N occurs through proteolysis and release of amino acids, amino acid deamination and release of ammonia, and from catabolism of other N-rich compounds stored in the vacuole (Bigot et al. 1991).

1.2.4 Role of the amino acids

Amino acids are defined as compounds containing a carboxyl and amino groups. Differences in their side-chain result in specific functional and biochemical properties. In addition to their role as structural constituents of proteins, the amino acids are central elements of the plant metabolism as precursors for primary and secondary metabolites (Facchini et al. 2000). Twenty types of amino acids constitute the basic unit of proteins (Wu 2009). Relative to plants supplied with 1 mM of NO_3^-, perennial ryegrass plants deprived of N for 10 days have presented a lower amino acid concentration in the root, especially for the N-rich amino acids glutamine and asparagine, and accumulated carbohydrate storage forms (Louahlia et al. 2008b). A metabolic profiling of *Solanum lycopersicum* revealed that plants transferred to N deficient medium (0.4 mM NO_3^-) for seven days were characterised by a rapid decline in aspartate, progressive decline of alanine and by a relatively less significant decline in arginine, glutamate, and tyrosine over time when compared to the initial concentrations before transfer. By contrast, tryptophan levels stayed high and lysine, and leucine increased throughout the experiment (Urbanczyk-Wochniak and Fernie 2005).

Individual amino acids modulate specifically cell membrane permeability and ion transport (Rai 2002). Phenylalanine, tyrosine, and tryptophan are classified as aromatic amino acids. They can be converted into a wide range of more complex aromatic compound such as lignin, alkaloids, and pigments, that play crucial roles in plant development (Siqueira et al. 1991), defence against herbivory (Unsicker et al. 2009), and environmental responses (Chalker-Scott 1999). The proteinogenic amino acid proline is the only amino acid to contain a secondary amine and to possess a cyclic structure which is associated with a relative conformational rigidity compared to the other amino acids. Proline is considered to be a metabolite with protective functions and accumulates under multiple environmental stress such as oxidative stress (Yang et al. 2009), drought (Choudhary et al. 2005), high salinity (Yoshiba et al. 1995), exposure to heavy metals (Schat et al. 1997), and biotic stresses (Fabro et al. 2004) in a conserved way across plant species (Szabados and Savoure 2010). Proline functions include radical scavenging (Wang et al. 2009), maintain of a redox balance (Islam et al. 2009), and protection of protein integrity (Sharma and Dubey 2005). In addition, proline could function as a signal molecule regulating gene expression (Szabados and Savoure 2010). Hence, contrasting NO_3^- availability is associated with specific regulations of the amino acid

metabolism, and results in fine regulations of metabolic networks and compounds from the secondary metabolism (Urbanczyk-Wochniak and Fernie 2005).

1.2.5 Regulation of nitrogen acquisition by nitrate and ammonium ions, and nitrogen by-products

Nitrogen uptake and assimilation are integrated to the nutritional need of the plant through feedback regulations by N itself and N by-products (Masclaux-Daubresse et al. 2010). Besides its role as a nutrient, NO_3^- is involved in processes such as balance of electric charges during ion flux (McClure et al. 1990), local and systemic signalling (Robinson and Rorison 1983, Ruffel et al. 2011, Alvarez et al. 2012), and a concentration gradient across the tonoplast which results in water entering in the vacuole by osmosis (Zirkle 1937). In *A. thaliana*, NO_3^- regulates the expression of numerous genes including those involved in N reduction (*NR*, *NiR*, genes coding enzymes involved in the GS-GOGAT pathway), as well as those involved in glycolysis, sugar metabolism, and photosynthesis (Scheible et al. 2004, Sakakibara 2006*a*). Split-root experiments in *A. thaliana* and perennial ryegrass plants suggested an induction of NO_3^- uptake systems by NO_3^- itself under N limiting conditions (Clarkson et al. 1986, Rawat et al. 1999, Gansel et al. 2001). An inverse relation is generally found between NO_3^- uptake and its internal concentration within the roots (Rawat et al. 1999). Intriguingly, recent evidence suggests that the NO_3^- transporter NRT1.1 acts at either low or high affinity, depending on the phosphorylation of threonine101 (Ho et al. 2009). In addition to acting as a transporter, NRT1.1 was identified as a receptor able to initiate local or systemic N signalling in *A. thaliana*, and is therefore referred to as a transceptor important for nutrient sensing (Ho et al. 2009).

Amino acids and N by-products are major constituents of plants saps. Cooper and Clarkson (1989) suggested that the amino acid content and forms cycling via the saps could act as a signal to the roots of the whole plant N status (Cooper and Clarkson 1989). External application of amino- and inorganic N sources has revealed that amino acids, especially glutamine and asparagine, are able to inhibit both NO_3^- and NH_4^+ uptake in perennial ryegrass (Thornton 2004, Thornton and Robinson 2005). Consistently, glutamine was shown to be a strong repressor of *NRT2.1* transcript in *A. thaliana* and could represent one of the main shoot-to-root N status signals regulating N uptake (Dechorgnat et al. 2011). In Figure

1.2, Glass et al. (2001) depict a model of regulation for one of the *A. thaliana* NO_3^- transporter coding gene *NRT2* and NH_4^+ transporter coding gene *AMT1.1* (Glass et al. 2001). While much remains to be understood, here in section 1.3 below, is highlighted the importance of a C/N balance in the control NUE.

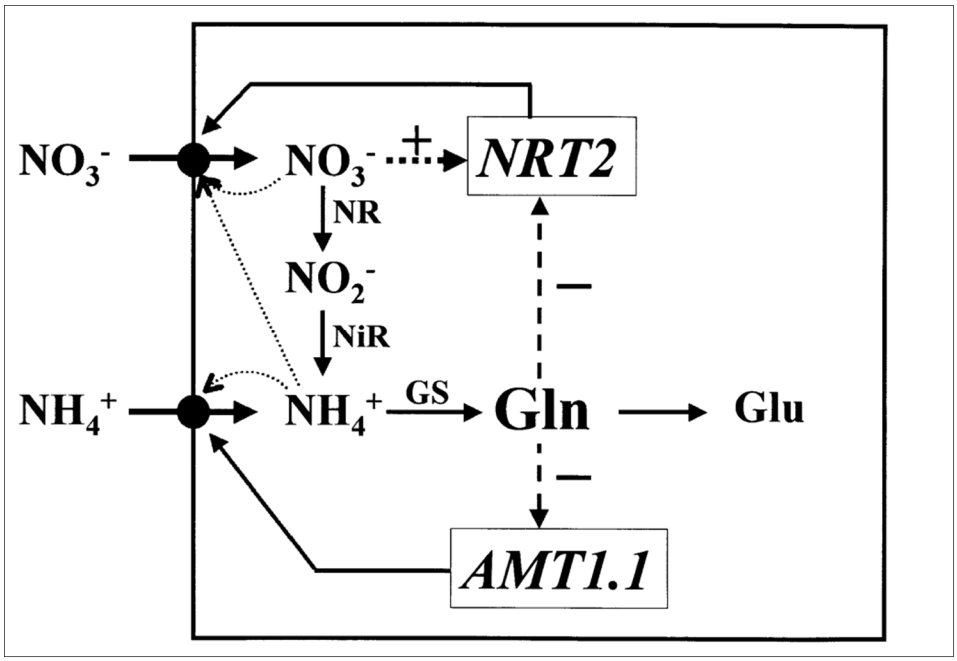


Figure 1.2. Model representing positive (+) and negative (-) feedback regulation of *NRT2* and *AMT1.1* transcripts by glutamine concentration (dashed line), and by nitrate (NO_3^-) and ammonium (NH_4^+) concentrations (square dots lines) in the root of *A. thaliana*. An increase in endogenous NO_3^- concentration results in up-regulation of *NRT2* gene expression and in NO_3^- reduction into NH_4^+ by action of the enzyme NO_3^- reductase (NR) and nitrite reductase (King et al.). Nitrogen assimilation is initiated by NH_4^+ conversion in glutamine by action of glutamine synthetase (GS). Nitrogen metabolites such as amino acids like glutamine negatively feedback regulates *NRT2* and *AMT1.1* transcript. Glutamine is converted in glutamate and other N-containing complex molecules. Figure obtained from Glass et al. (2001).

Abbreviations: AMT, ammonium transporter Gln, glutamine; Glu, glutamate; GS, glutamine synthetase; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate transporter.

1. 3 Water-soluble carbohydrate remobilisation controls nitrogen assimilation and subsequent growth response

1.3.1 Lag phase between nitrogen supply and growth response

In a previous research work at the University of Canterbury, Dawar et al. (2010) measured perennial ryegrass NUE at 21 and 42 days after N treatments, which corresponded to an on-farm fertiliser and grazing cycle (Fig. 1.3) (Dawar et al. 2010).

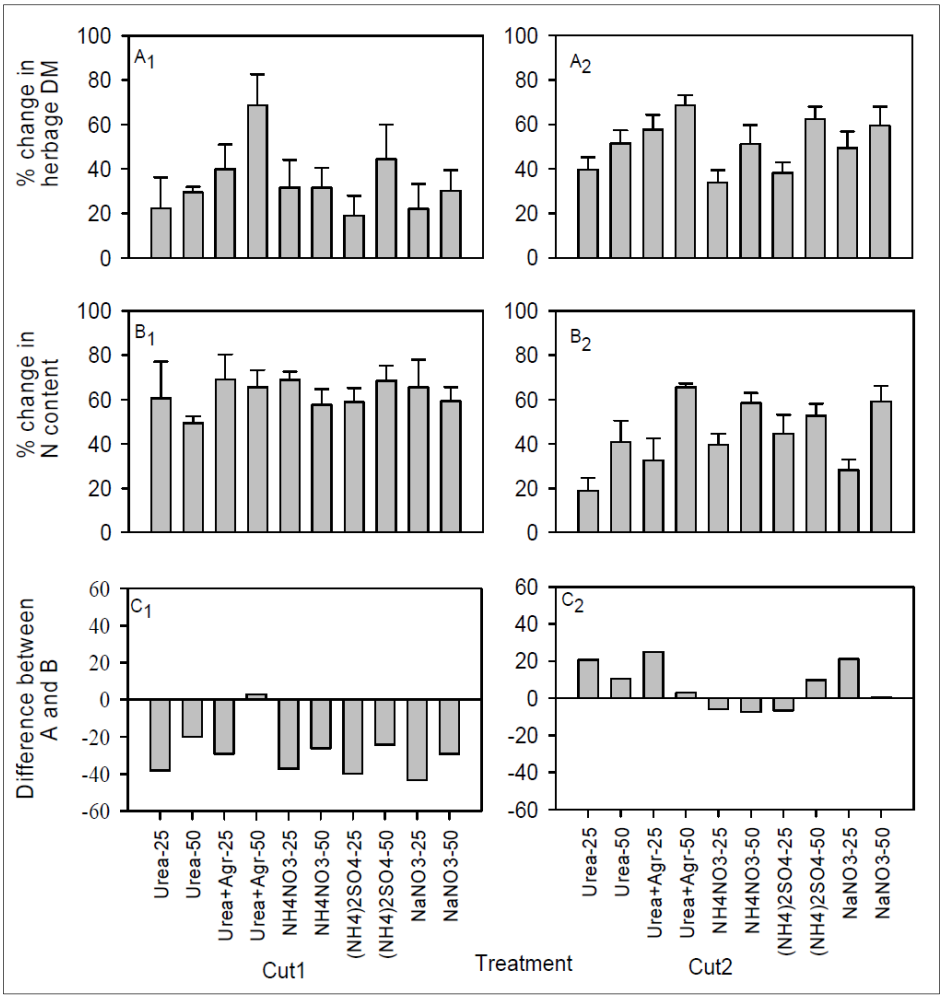


Figure 1.3. Change in herbage dry matter (DM) (A), nitrogen content (B) and difference between change in herbage DM and nitrogen content (C), at cutting, 21 days (1) and 42 days (2) after treatment with different nitrogen fertiliser forms. Data are means ±SE, n=4. Statistical analysis were performed by analysis of variance (Kavanova et al.) and least significant differences (LSD) were calculated to compare treatment means when $p < 0.05$. Reproduced from Dawar et al. (2010), with permission from CSIRO publishing.

Dawar et al. (2010) noted an overall greater N content at cut 1 (Fig. 1.3B1) compared with cut 2 (Fig. 1.3B2) across most fertiliser forms, indicating that N uptake was greater during the initial 21 days after cut rather than between 21 and 42 days after cut. The difference between change in DM and change in N content was negative at cut 1 (Fig. 1.3C1) and positive at cut 2 (Fig. 1.3C2), which indicates that N taken up was not fully assimilated by 21 days after cut, whereas by 42 days after cut the growth increment had equalled or exceeded the change in N content (Dawar et al. 2010). Dawar et al. (2010) suggested that the lack of a strong growth response early in the pasture cycle may limit the efficient use of applied N. This study highlights the need for a better understanding of the mechanism taking place following N uptake and which limited the subsequent growth response.

1.3.2 Water-soluble carbohydrate supplies carbon skeletons for nitrogen assimilation

Nitrogen use relies on carbohydrate breakdown to supply C skeletons for N assimilation, ATP for energy, and production of reducing equivalents (Krapp and Traong 2006). Conversely, C metabolism requires enzymatic activity and the coordinated use of N to build cell structural components and to support growth (Zheng 2009).

Non structural carbohydrates are the major constituent of C reserves and include reducing sugar such as glucose and fructose, non reducing sugar such as sucrose, fructosan, and starch (White 1973). Most plants accumulate C reserve as polymers of glucose, forming starch (Turner et al. 2006). However, fructans are the main storage form of water-soluble carbohydrate (WSC) in grasses and cereals, including perennial ryegrass (Turner et al. 2006).

Not only are WSCs a highly accessible energy source providing C for N assimilation (Weinmann 1947), they also are able to support regrowth following defoliation (Donaghyē and Fulkersonǵ 1998) and, additionally, are an important nutritional element of the pasture (Danckwerts and Gordon 1987, Chalmers et al. 2005).

The WSC reserves are generally synthesised and accumulated within the vacuoles of cells located at the tiller base, which includes mature leaf sheaths and elongating leaf bases (Pollock and Cairns 1991, Vijn and Smeekens 1999, Cairns and Gallagher 2004). The

dynamics of WSC accumulation and remobilisation are critical components for N assimilation and involve species specific enzymatic activities (Hisano et al. 2004, Chalmers et al. 2005, Lothier et al. 2007). The biosynthesis of linear and branched fructans involves at least four fructosyltransferase enzymes in perennial ryegrass: sucrose-sucrose 1-fructosyltransferase, sucrose-fructan 6-fructosyltransferase, fructan-fructan 1-fructosyltransferase and fructan-fructan 6G-fructosyltransferase (Hisano et al. 2004). In addition, Lothier et al. (2007) suggested that fructan exohydrolase enzymes, which have been characterised as fructan breakdown enzymes, might also play a role in WSC synthesis (Lothier et al. 2007). The inulin series, inulin neoseries, and levan neoseries are the three fructan series found in perennial ryegrass based on their physical structure (Pavis et al. 2001).

The C/N balance relies on adjustments of the NO_3^- uptake rate and assimilation to the maintenance of WSC availability or remobilisation (Price et al. 2004, Krouk et al. 2009, Tschoep et al. 2009). Remobilisation of WSCs occurs when photosynthetic C production does not meet the C demand, e.g., in response to N treatment (McIntyre et al. 2011), at night time (Waite and Boyd 1953), or following defoliation (Ourry et al. 1990b). Treatment with N fertiliser is associated with an increase in C-demand and results in the subsequent remobilisation of WSCs reserve by hydrolysis (Morvan-Bertrand et al. 1999, Grechi et al. 2007).

1.3.3 Dynamics of carbon remobilisation as affected by diurnal variations and during regrowth following defoliation

Morvan-Bertrand et al. (1999) showed that four days of exposure to contrasting photoperiodic conditions (darkness, 16 h day: 8 h night cycle, or continuous light) as an experimental treatment resulted in differential accumulation of carbohydrate stores and rapidly affected NUE of perennial ryegrass plants (Morvan-Bertrand et al. 1999). Genome-wide analysis revealed that N uptake stimulated photosynthetic capacity of the plant and stimulated leaf growth, thereby increasing the capacity to supply C for rapid N use (Puig et al. 2012).

Diurnal variations are associated with tissue specific uses of the WSCs pool: during day light, photosynthetically derived C supports direct leaf growth and any excess C is exported to sink tissues or stored as more complex forms of WSCs, whereas darkness is associated with a halt

in leaf growth. By contrast, leaf sheath, and root tissues are continuously fuelled by C import from the phloem (Lattanzi et al. 2005, Huanosto Magana et al. 2009). The overall increase in availability of the simple sugars produced via photosynthesis during day light correlates with a decrease in internal concentrations of NO_3^- and with an increase in free amino acids content, and recovery of the WSCs stores (Louahlia et al. 2008b). By contrast, limitation of N availability in the environment is usually associated with the accumulation of WSCs stores and decreased concentrations of amino acids, especially the amino acid presenting a high C/N ratio such as arginine, glutamine and histidine (Nowakowski 1962, Fritz et al. 2006). Exogenous treatments with the mono- and di-saccharides glucose, sucrose or fructose to the roots of high-N perennial ryegrass plants rapidly stimulated NO_3^- uptake (Louahlia et al. 2008a).

In addition to diurnal variations, defoliation of perennial ryegrass plants results in strong remobilisation of the WSCs (Wang et al. 2012a). Rapid regrowth after defoliation relies on complementary fluxes of newly-acquired and reserve-derived C and N, and is associated with re-establishment of a balanced C/N ratio from complementary fluxes of reserve-derived and currently assimilated C and N (Richards and Caldwell 1985, Ourry et al. 1989, Schnyder 1999). Foliage production rate after defoliation is related to C rather than N supply (Schnyder 1999).

Prud'homme et al. (1992) identified two-phases in WSC dynamics following defoliation - the WSCs are remobilised in all tissues for up to six days following defoliation to sustain foliage development, after which a second period is characterised by a recovery of the WSCs stores levels for up to 29 days after defoliation (Prud'homme et al. 1992). In addition, C can be used directly from photosynthesis of early regrown tissues, with a proportional increase in photosynthate use compared with WSC reserve remobilisation over time following defoliation as more photosynthetic tissues develop (Morvan-Bertrand et al. 1999).

Ourry et al. (1990) showed that N taken up after defoliation was not directly assimilated but stored mainly in the vacuole of the leaf, sheath and root tissues for up to five days after defoliation. This was followed in time by a period of N reduction throughout the plant between five and 12 days after defoliation (Ourry et al. 1990a). Lestienne et al. (2006) noted that an increase in defoliation intensity correlated with greater amount of N taken up and directly transported from root to growing leaves relative to the amount of N taken up, and

allocated to roots and other shoot parts (Lestienne et al. 2006). Therefore, N use following defoliation is influenced by the initial WSC availability and by N mobilisation through proteolysis and/or amino acids hydrolysis (Ourry et al. 1989, Morvan-Bertrand et al. 1999).

Guo et al. (2016) noted that defoliation resulted in down-regulation at the transcriptional level of perennial ryegrass NO_3^- transport system and changes NO_3^- assimilation, whereas an exogenous treatment with 1% glucose was able to rescue the reduced nitrate uptake (Q Guo, University of Canterbury, School of Biological Sciences, Christchurch, New Zealand, unpubl. res.).

Attempts to identify 'high-sugar' perennial ryegrass cultivars by plant breeders resulted in significantly greater content of WSCs, although inconsistency was observed in WSCs contents following defoliation (Rasmussen et al. 2009). Alternatively, perennial ryegrass plants were produced by transgenesis of perennial ryegrass lines with onion (*Allium cepa* L.) sucrose-sucrose 1-fructosyltransferase and fructan-fructan 6G-fructosyltransferase in order to obtain forage crops with improved forage quality (Gadegaard et al. 2008, O'Callaghan et al. 2010). This transgenic lines exhibited up to a three-fold increase in fructan content and stable fructan levels during the growth period relative to control lines of perennial ryegrass (Gadegaard et al. 2008).

Plants responses to C/N imbalance must be coordinated at the whole-plant level and involves both local and systemic root-shoot-root signals (Sakakibara 2006*a*). The following section focusses on the cytokinins as they have been implicated in communicating the nutrient status between organs.

1. 4 Cytokinins

1.4.1 Cytokinins controls over plant development

The cytokinins are a class of plant hormone that were initially identified as a diffuse factor that positively regulates cell division and was initially referred to as "kinetin" (Miller et al. 1955). The first cytokinin identified from plants were isolated from *Zea mays* endosperm in the 1970s (Letham 1973). Since then, the cytokinin have been shown to control many aspects of plant growth and development with a specificity of actions varying upon tissue types and

in a dose-dependent manner (Shibaoka 1994, Vissenberg et al. 2000, Sakakibara 2006*a*). The signal associated with cytokinins was found to be implicated in nutrient use (Roitsch and Ehneß 2000), lateral shoot growth (Moubayidin et al. 2009), shoot and root apical dominance and meristematic activity (Pospíšilová et al. 1997), as well as leaf senescence (Gan and Amasino 1997), floral development (Faiss et al. 1997), bud dormancy breaking (Pospíšilová et al. 2000), seed germination (Jameson and Song 2016), and light-regulated development (Sakakibara 2006*a*, Mazid et al. 2011).

Exogenous cytokinins treatment to *A. thaliana* plants resulted in a reduced root meristem size via an increase in cell differentiation (Ioio et al. 2007, Kuderová and Hejátko 2009, Garay-Arroyo et al. 2012). In contrast, lower cytokinin content in transgenic plants of *A. thaliana* overexpressing the cytokinin oxidase/dehydrogenase gene family was associated with longer roots relative to the wild-type (Werner et al. 2003). Indeed, under sufficient homogeneous NO_3^- supply, cytokinins regulate the synthesis and transport of the plant hormone auxin (Su et al. 2011). Together, auxin and cytokinin can regulate the organisation of the actin filament and drive directional changes in root growth (Kushwah et al. 2011).

In contrast to root growth inhibition, cytokinins promote cell division and differentiation in the shoot, and an increase in cytokinin levels in the xylem sap correlated with an increase in leaf expansion rate (Takei et al. 2001, Rahayu et al. 2005). Kavanova et al. (2008) investigated perennial ryegrass leaf growth under contrasting N supply and found that N deficiency (1 mM NO_3^-) correlated with a decrease in leaf elongation by 43% relative to the plants supplied with sufficient N (7.5 mM NO_3^-). Despite a lack of data for perennial ryegrass plants, current knowledge developed in *A. thaliana* plants suggests a link between a reduced cytokinin content under N deficiency and reduction in leaf elongation (Takei et al. 2001, Kavanova et al. 2008).

1.4.2 Cytokinin metabolic and signalling pathways

Although collectively referred to as cytokinins, functional differences have been associated with significant variations in the cytokinin side-chain structure of these N(6)-substituted adenine derivatives (Hothorn et al. 2011). The most common cytokinins found in higher plants are isopentenyladenine (iP), *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), and dihydrozeatin (DZ), which differ in the presence and stereoisomeric position of a hydroxyl group at the end of the

prenyl side chain (Mok and Mok 2001). Two distinct pathways have been proposed for cytokinin biosynthesis. The ADP/ATP pathway is considered to be the major pathway, whereas the tRNA pathway account for a limited amount of cytokinins due to slow turnover rate of tRNA (Haberer and Kieber 2002). The *c*Z- and iP-type cytokinins are normal constituents of certain tRNAs (Sakakibara 2006*a*). Schäfer et al. (2015) suggested that various stress conditions induce tRNA turnover pathways. They noted that *c*Z-containing tRNA can be formed from hydroxylation of the iP-containing tRNA and that the action of an unknown enzyme on these tRNAs could release *c*Z/*c*ZR forms and iPR forms, respectively (Schäfer et al. 2015). Gajdošová et al. (2011) suggested that *c*Z and/or its derivatives might maintain minimal levels of cytokinin under growth-limiting conditions necessary for plant survival and subsequent recovery (Gajdošová et al. 2011).

Cytokinins have been classified according to their physiological functions into active, transported, storage, and inactivated forms (Sakakibara 2010). In *A. thaliana*, the free-base cytokinins iP and *t*Z are actively perceived by the membrane-located cytokinin receptors, respectively CRE1/AHK4 and AHK3 (acting together with AHK2), and are considered the biologically active forms (Inoue et al. 2001, Yamada et al. 2001, Spíchal et al. 2004, Lomin et al. 2015), whereas *c*Z and DZ affinities are more than one order of magnitude lower in *Escherichia coli* (Stolz et al. 2011). The nucleotides are identified as early biosynthetic forms, the nucleosides as translocation forms and glucosides as storage or inactivated forms (Spíchal 2012).

Following recognition of the accumulating cytokinin free-bases by their receptors (Lomin et al. 2015), a downstream signalling cascade is initiated by a His-Asp phosphorelay system between two types of signal transducer, typically a sensor histidine kinase and a response regulator (RR), and is therefore referred to as the ‘two-component system’ (Hwang and Sheen 2001). The early cytokinin signalling events include an initial autophosphorylation of a histidine kinase and phosphorelay transfer to a receiver domain of RR (Yamada et al. 2001) resulting in modification of RR ability to act as transcription factor (Sakai et al. 2001) and subsequent increase in expression of *RRs* (Sakakibara et al. 1998).The Type-B RRs serve as direct transcriptional regulators for certain target genes, including Type-A *RRs* (Jain et al. 2006). A negative feedback regulation exists between Type-B and Type-A *RRs* (Dortay et al. 2006, Sakakibara 2006*a*).

1.4.3 Cytokinin interplay with nitrogen metabolism

Nitrogen status of the plant can be communicated by NO_3^- specific signalling controlling synthesis of amino acids, C metabolism and hormonal homeostasis, and by cytokinin mediated signalling mainly regulating N partitioning (Roitsch and Ehneß 2000, Wang and Ruan 2016), and plant development (Takei et al. 2001, Forde 2002, Sakakibara 2003).

Sattelmacher and Marschner (1978) were the first to show a relation between N nutrition and cytokinin activity in *Solanum tuberosum*, and revealed an increase in cytokinin activity under N withdrawal (Sattelmacher and Marschner 1978). Subsequently, Horgan and Wareing (1980) identified a correlation between shoot growth response to N deficiency and levels of cytokinin free-bases and nucleosides (Horgan and Wareing 1980). Nitrate addition to N-deficient plants up-regulates the specific gene family members coding for *A. thaliana* (*AtIPT3*) (Miyawaki et al. 2004, Takei et al. 2004) and rice (*OsIPT4, OsIPT5, OsIPT7, OsIPT8*) isopentenyl transferase (the rate limiting enzyme in cytokinin biosynthesis) (Kamada-Nobusada et al. 2013).

Nitrate-mediated cytokinin accumulation appears to be a mechanism conserved among higher plants and has been shown inter alia in *A. thaliana* (Takei et al. 2002), *Z. mays* (Sakakibara et al. 1998), rice (Kamada-Nobusada et al. 2013), barley (Samuelson and Larsson 1993), wheat (Garnica et al. 2010), tobacco (Singh et al. 1992), a perennial herb *Urtica dioica* (Wagner and Beck 1993), and perennial ryegrass (Wang et al. 2013). Consistently, *A. thaliana* seedlings grown with a high concentration of NO_3^- contain higher levels of cytokinins than those grown with a low NO_3^- supply (Kiba et al. 2011). Takei et al. (2004) suggested that N availability differentially regulates the expression of *AtIPT3* and *AtIPT5* and that *AtIPT3* is a key determinant of NO_3^- dependent cytokinin biosynthesis in *A. thaliana*. In addition, up-regulation of Type-A *RRs* by NO_3^- was shown in both maize (Sakakibara et al. 1998) and *A. thaliana* (Taniguchi et al. 1998).

Recently, Shtratnikova et al. (2015) showed that NO_3^- and not NH_4^+ increased the expression of *A. thaliana* Type-A *RR5::GUS* reporter constructs. While NH_4^+ is the main inorganic N source for paddy rice (Kamada-Nobusada et al. 2013), NH_4^+ salts applied alone reduces growth of many plants. However, this negative effect can be ameliorated by application of very low NO_3^- concentrations (Rahayu et al. 2005) or by cytokinin (Shtratnikova et al. 2015),

with the conclusion that it is the lack of cytokinin, not NO_3^-, limiting growth in plants supplied with NH_4^+ as sole N source (Shtratnikova et al. 2015).

Exogenous application of cytokinin to *A. thaliana* roots down-regulates the expression of amino acid, NH_4^+ and NO_3^- uptake transporter genes (Fan et al. 2009). Most shoot-expressed NO_3^- transporter genes are up-regulated by cytokinins under both high- and low- N conditions, whereas cytokinins can down-regulate root transporters such as *AtNRT1.1*, *AtNRT2.1* and *AtNRT2.2*, and *NRT1.7* gene coding for transporter implicated in the remobilisation of NO_3^- from root to shoot (Brenner et al. 2005). This suggests that cytokinins could enhance NO_3^- distribution and translocation from the root to the shoot (Fan et al. 2009). In addition, cytokinins have been implicated in the regulation of *NRT* genes, N assimilation genes (*NRT*, *NR*, *AS*, and glutamine dehydrogenase), and C metabolism (Sakakibara et al. 2006*b*).

In *A. thaliana*, microarray analysis following addition of NO_3^- and cytokinin showed that N and/or cytokinin are able to regulate a variety of genes involved in NUE and C metabolism, and more particularly in processes such as NO_3^- uptake and reduction, ammonium assimilation, amino acid metabolism, carbohydrate metabolism, glycolysis, cytokinin homeostasis and signalling (Fig. 1.4) (Brenner et al. 2005, Sakakibara et al. 2006*b*). In addition, plant photosynthetic capacity is under the regulation of both NO_3^- and cytokinin signals via regulation of genes coding enzymes involved in trehalose metabolism (Brenner et al. 2005).

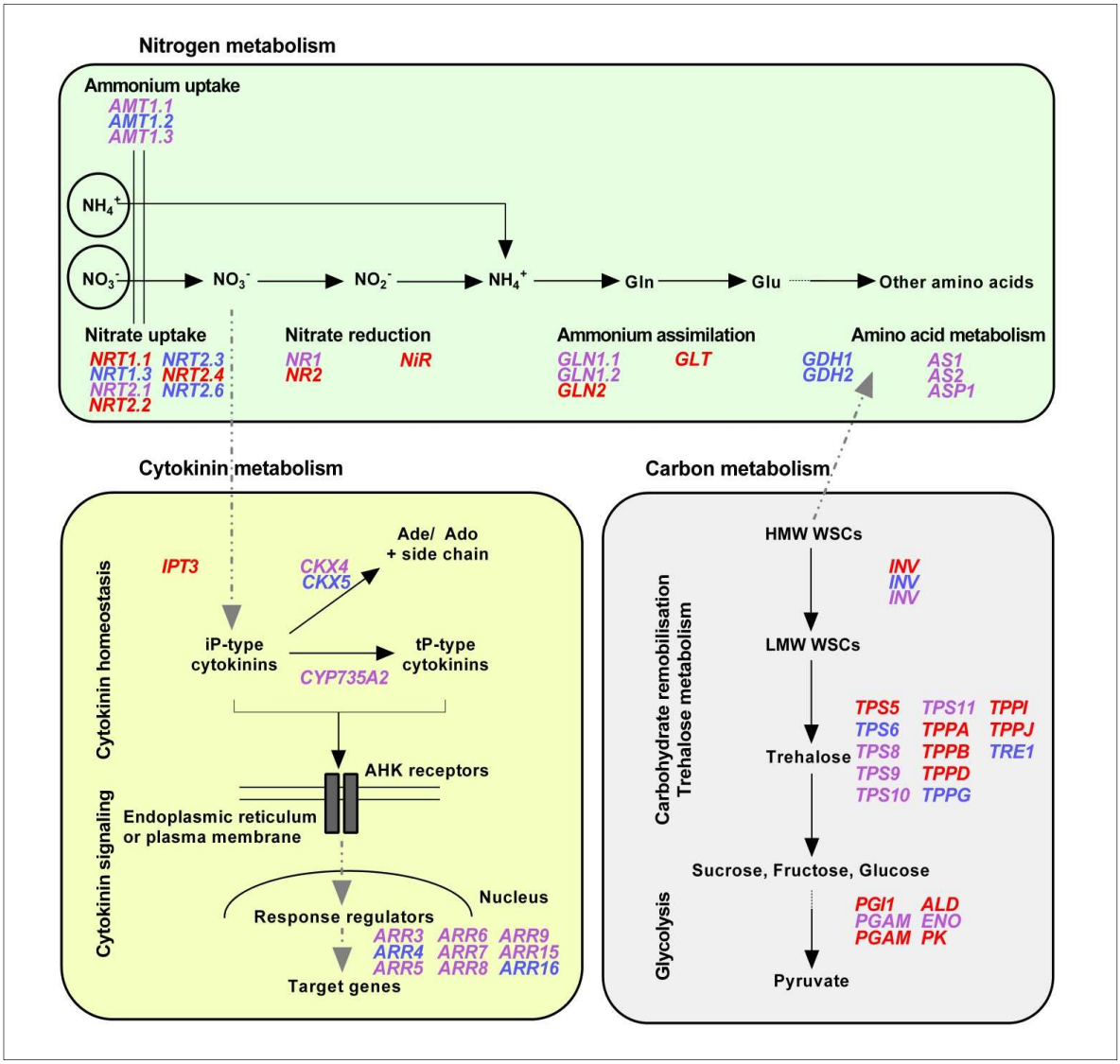


Figure 1.4. Regulation of genes involved in nitrogen, cytokinin and carbon metabolism by nitrate (red), cytokinin (blue), and by both nitrate and cytokinin (purple) in *Arabidopsis thaliana* plants. Dashed arrows represent nitrate up-regulation of cytokinin biosynthesis genes and carbon supply for nitrogen assimilation and amino acid biosynthesis. Abbreviations: AMT, ammonium transporter; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate transporter; GLN, glutamine synthetase; GLT, NADH-glutamate synthase; GDH, glutamate dehydrogenase; ASP, aspartate aminotransferase; AS, asparagine synthetase; IPT, isopentenyltransferase; CKX, cytokinin oxidase; CYP735A2, cytokinin *trans*-hydroxylase; AHK, *A. thaliana* histidine kinase; ARR, *A. thaliana* cytokinin response regulator; HMW WSCs, high molecular weight water-soluble carbohydrates; LMW WSCs, low molecular weight water-soluble carbohydrates; INV, invertase; TPS, trehalose-6-P synthase; TPP, trehalose-6-P phosphatase; TRE, trehalase; PGI, glucose-6-P isomerase; PGAM, phosphoglycerate mutase; ALD, aldolase; ENO, enolase; PK, pyruvate kinase. Modified from Sakakibara (2006*b*).

Takei et al. (2001) described an initial accumulation of cytokinins in the root followed on time by accumulation in xylem sap and leaves and ultimate induction of *ZmRR1* gene expression during the resupply of N to *Z. mays* (Takei et al. 2001). Indeed, NO_3^--mediated cytokinin induction results in an increase in the non-hydroxylated cytokinins (referred to subsequently as iP-type cytokinins) in the root and subsequent conversion predominantly in the root and stem to hydroxylated forms (referred to subsequently as *t*Z -types) (Sakakibara et al. 2006*b*). The *t*Z -type cytokinins are generally found in the xylem and could be a root to shoot signal of NO_3^- availability regulating shoot growth (Takei et al. 2001, Kiba et al. 2013), whereas the iP-type cytokinins are mainly detected in the phloem and might play a role as a shoot to root signal of the whole plant N status (Hirose et al. 2008, Ruffel et al. 2011, Kiba et al. 2013). Consistently, the translocation rate of cytokinin from root to shoot appears to be dependent upon the N condition and this mechanism is conserved among higher plants, e.g in *Z. mays* (Takei et al. 2001), *Urtica dioica* perennial herb plants (Wagner and Beck 1993) and *P. sativum* (Beveridge et al. 1997).

In addition to unidirectional long-distance cytokinin signalling between root and shoot (Stolz et al. 2011). Ruffel et al. (2011) showed that cytokinins could be crucial components of a systemic root-shoot-root signal integrating the NO_3^- status of the plant (Kiba et al. 2011, Ruffel et al. 2011, Stolz et al. 2011). In a split-root experiment exposing cytokinin biosynthetic mutants to contrasting NO_3^- treatments Ruffel et al. (2011) identified that genetically independent local and root to shoot to root systemic signals controlling local root growth under heterogeneous N soil conditions (Ruffel et al. 2011).

Wang and Ruan (2016) proposed a model in which N limitation results in cytokinin deficiency which may promote C allocation from shoot to root, subsequent root growth, thereby strengthening a N/cytokinin positive feedback loop resulting in N uptake and assimilation and associated cytokinin systemic signal balance the C/N ratio and ultimately plant root/shoot growth (Wang and Ruan 2016). Further work is required to better understand this complex hormonal control loop and determine how changes in C and N are integrated to regulate growth of perennial ryegrass.

1. 5 Aims and objectives

A more science-informed management of N fertiliser application and grazing cycles is required in order to increase crop productivity and profitability while decreasing the environmental impact of the *L. perenne* production system. Plant growth is a multicomponent process resulting from the uptake of nutrients, associated local and systemic signals, maintain of homeostasis and ultimate physiological responses (Durbak et al. 2012). Key gaps in current understanding identified in the review above are: the extent of the transfer of knowledge from the model plant *A. thaliana* to *L. perenne*, the synchronising of WSCs remobilisation with NO_3^- assimilation (see sections 1.3.2 and 1.3.3 above), the molecular events taking place between NO_3^- assimilation into amino acids and N-by products and subsequent growth response (see section 1.2.5, 1.3.1 and 1.3.2), the characterisation of cytokinin profiling associated with N use in perennial ryegrass plants (see section 1.4.3), as well as a physiological and molecular understanding of the effect of grazing on N and C metabolism (see section 1.3.3).

This research project aims at providing an integrated spatio-temporal dynamic of NO_3^- assimilation, maintain of C/N balance and cytokinin signalling in perennial ryegrass plants. Three main research questions were investigated.

1. What are the spatiotemporal metabolic changes taking place over the seven days following NO_3^- treatment to perennial ryegrass plants?
Changes in WSCs, amino acids, tricarboxylic acid cycle intermediates and associated cytokinin signals were recorded in response to NO_3^- assimilation in roots and shoots of *L. perenne* following treatment with high concentration of NO_3^- (5 mM) of plants previously grown for 10 weeks in unfertilised soil. Metabolomic analysis by GC-MS and LC/MS-MS, gene expression and hormonal content were described in Chapter 2. The findings of this chapter have been published in *Physiologia plantarum*. DOI: 10.1111/ppl.12412. A copy of the paper can be found in the Appendix 2.

2. How does defoliation-induced increase in C-demand affect the coordinated use of C and N for efficient NO_3^- assimilation and subsequent regrowth over a period of seven days following defoliation?

The effect of perturbations of the WSC availability on N assimilation was tested on plants exposed to either continuous light or short days (8 h light : 16 h dark) and to either high (5 mM) or low (50 µM) NO_3^- as a sole N source and stressed with an increase in C demand experimentally induced by a defoliation treatment in perennial ryegrass plants. Chapter 3 presents an integrated analysis of the interaction between N and C metabolism by measurements of leaf, sheath and root WSCs remobilisation, amino acid profiling and associated sheath/root cytokinin signals during early regrowth of *L. perenne*. The finding of this chapter have been submitted in *Annals of Botany* and is currently been reviewed by the journal editors.

3. Is it possible to perturb perennial ryegrass NUE by exogenous application of a cytokinin-like compound and cytokinin antagonist?

Perennial ryegrass plants were treated with the synthetic compounds CPPU and PI-55, with the hypothesis that CPPU would increase the endogenous cytokinin levels and PI-55 would inhibit cytokinin signalling pathway. A dose-response relationship to CPPU applications was estimated by physiological measurements of biomass, tiller number, leaf number, leaf regrowth rate, maximum photosynthetic rate A_{max} and stomatal conductance g_s over a 28 day period.

The activation of cytokinin signalling pathway in response to 5 mM KNO_3 treatment was estimated by monitoring the relative expression of Type-A and Type-B *LpRR* gene family members following PI-55 treatment hydroponically. Results are summarised in Chapter 4.

Finally, the findings described in Chapters 2, 3 and 4 are synthesised and further paths of study are suggested in Chapter 5.

Chapter 2

Metabolic changes and associated cytokinin signals in response to nitrate assimilation in roots and shoots of *Lolium perenne* L.

Contents

Roche J, Love J, Guo Q, Song J, Cao M, Fraser K, Huege J, Jones C, Novák O, Turnbull MH and Jameson PE (2016) Metabolic changes and associated cytokinin signals in response to nitrate assimilation in roots and shoots of Lolium perenne. *Physiologia plantarum* **156**:497-511. (Appendix 2)

2. 1 Introduction

Nitrogen (N) availability is a major limiting factor to plant growth and crop production (Dinnes et al. 2002) (see section 1.1 for background). In modern crop management systems, production of forage grass for grazing stock is maintained by exogenous application of N fertiliser supplied as urea, nitrate (NO_3^-) or ammonium (NH_4^+) (Robertson and Vitousek 2009). Excessive use of N fertiliser is associated with negative long-term effects and improving plant N use efficiency is necessary to limit the impacts on the environmental (see sections 1.1 and 1.2.1). Nitrogen use efficiency can be defined as total biomass produced per unit of applied N fertiliser and corresponds to the sum of the efficiency of N uptake from the exogenous environment and N assimilation efficiency (see section 1.2.1) (Good et al. 2004, Hirel et al. 2007, Masclaux-Daubresse et al. 2010). Various amino acids, nucleic acids, and other N-derived secondary metabolites are produced as a result of NO_3^- assimilation with carbon (C)-accepting molecule (see sections 1.2.3, 1.3.2). Consequently, the efficiency of N assimilation depends directly on C metabolism to supply C skeleton from breakdown of water soluble carbohydrate (WSC) reserves, ATP for energy, and reducing equivalent (see sections 1.2.3, 1.3.2, 1.3.3) (Krapp and Traong 2006). Although the metabolic response of perennial ryegrass to N addition is clearly a complex one, metabolome analysis to investigate N assimilation in perennial ryegrass has not yet been broadly used. The cytokinins are a class of plant hormone which control many aspects of plant growth and development (see section 1.4.1) (Sakakibara 2006). Ruffel (2011) hypothesised that cytokinins act as a local NO_3^- signal and as a systemic root-shoot-root signal integrating the NO_3^- status of the plant (see sections 1.4.1, 1.4.3) (Ruffel et al. 2011). Up to now, the molecular mechanisms and regulation of NO_3^- assimilation, its interaction with C metabolism, and involvement of cytokinin signals have been studied mostly in *Arabidopsis thaliana*, and continued efforts to further understand these processes are necessary to achieve an improvement of N use efficiency in perennial ryegrass (*Lolium perenne*) pasture systems.

The aim of the work presented in this chapter and published as (Roche et al. 2016) was to develop a spatiotemporal understanding of the events taking place during early NO_3^- assimilation in perennial ryegrass, i.e., between NO_3^- supply and subsequent N-associated growth response of young plants grown previously under steady-state low-N availability (see sections 1.2.1, 1.3.1, 1.3.2, 1.4.3 and 1.5). Of particular interest was the interaction between

N- and C- metabolism in different tissues (see section 1.3.2), and the putative involvement of cytokinin in perennial ryegrass (see sections 1.4.2 and 1.4.3). The analyses included the physiological response, metabolic profile, and gene expression in the roots and the shoots of 10-week old perennial ryegrass plants grown on unfertilised soil and subsequently exposed to high levels (5 mM) of KNO_3 for up to seven days. The metabolic analysis included a profiling of the WSCs across their degrees of polymerisation, and a profiling of the amino acids. Cytokinin content was measured in response to 5 mM KNO_3 or KCl treatment of steady-state N-deficient perennial ryegrass plants, and the expression of cytokinin response regulator genes *LpRR* was monitored by RT-qPCR, with the expectation that perennial ryegrass possesses a cytokinin-mediated N-signalling/relay system similar to that characterised in model plant species.

2. 2 Material and methods

2.2.1 Plant material

Lolium perenne L. cv. "Grasslands Nui" seedlings were grown for 10 weeks in a pot experiment conducted at the University of Canterbury glasshouses (43^{O}31'48" S, 172^{O}37'13" E) using unfertilised bark-free soil commercially acquired near Christchurch, New Zealand. The soil component analysis is presented in Table 2.1. Single perennial ryegrass seeds were sown directly onto perlite filling a 1.5 mL Eppendorf tube, the bottom of which had previously been cut off. The tubes with the single seed derived plant were placed on individual pots until plant establishment. Soil water content was maintained by automatic watering for five minutes daily by mist emitters located throughout the room. Roof ventilation was set to maintain 22^{O}C temperature. Once established for two months, plants were removed from their pots, the roots were washed, and their tubes were placed into fitted holes within polyvinyl chloride channels in a homemade hydroponic system. Seedlings were maintained for one week in Hoagland -N liquid medium (N-free Hoagland medium, BioWorld, USA) for acclimation to liquid growth conditions.

Treatments with 5 mM KCl or KNO_3 defined day zero (d0) of the experiment, and were performed by direct addition into the liquid medium after a week-long acclimation period. Temperature, pH, and electrical conductivity of the medium were monitored daily and, when required, the water level and pH were adjusted. Perennial ryegrass roots and shoots were destructively harvested on days one (d1), three (d3), and seven (d7), and the plant material

was immediately flash frozen in liquid N and subsequently stored at -80^{O}C. Tissue samples harvested from five plants were pooled and subsequently treated as one biological replicate. Total N and total C content of roots and shoots were quantified by Isotope Ratio Mass Spectrometer (IRMS) at the Department of Soil and Physical Sciences, Lincoln University, Christchurch, New Zealand. Three biological replicates were analysed.

2.2.2 Water soluble carbohydrates. tricarboxylic acid intermediates, and amino acids analysis

Tissue samples harvested from five plants were ground under liquid N and subsequently treated as one biological replicate. Samples were freeze-dried for three days. Each sample was weighted to 250 mg DW and the aliquots were sent to AgResearch Grasslands Research Centre (Palmerston North, New Zealand) for further analysis. Water-soluble carbohydrates of low and high molecular weight were extracted and subjected to LC-MS analysis as described in Harrison et al. (2009). Oligosaccharides of degree of polymerisation (DP) from DP2 to DP35 were quantified based on the extracted ion chromatogram (XIC) using methods largely described in Cao et al. (2013) (see Appendix 2.1 for full detail of the procedure).

2.2.3 Cytokinin measurements

Tissue samples harvested from five plants were ground under liquid N, freeze-dried, and aliquoted to 3 to 5.5 mg DW at the University of Canterbury (Christchurch, New Zealand). Pooled samples from five plants were subsequently treated as one biological replicate. Prepared biological triplicates were extracted and purified at the Laboratory of Growth regulators and Department of Chemical Biology and Genetics (Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University, and Institute of Experimental Botany, Olomouc, Czech Republic) using the method published previously by Dobrev and Kamínek (2002) with some minor modifications (Antoniadi et al. 2015) (see Appendix 2.2 for full detail of the procedure).

Table 2.1. Physical and chemical properties of the soil.

Soil properties	Unit	Level Found	Medium range	Low Medium High
pH	pH Units	7.1	5.8 - 6.2	
Olsen Phosphorus P	mg L^{-1}	11	20 - 30	
Potassium K^{+}	me $100g^{-1}$	0.16	0.4 - 0.6	
Calcium Ca_2^{+}	me $100g^{-1}$	6	4 - 10	
Magnesium Mg_2^{+}	me $100g^{-1}$	0.74	1 - 1.5	
Sodium Na^{+}	me $100g^{-1}$	0.12	0.2 - 0.5	
Cation exchange capacity	me $100g^{-1}$	8	12 - 25	
Total base saturation	%	90	50 - 85	
Volume weight	g mL^{-1}	1.24	0.6 - 1	
Total carbon	%	1.6		
Dry matter	%	81.5		
Moisture	%	18.5		
Ammonium-N	mg kg^{-1}	< 1		
Nitrate-N	mg kg^{-1}	5		
Mineral N	mg kg^{-1}	6		
Organic Matter	%	2.8	7 -17	
Base Saturation	%	K 2.1	Ca 77	Mg 9.5 Na 1.5

2.2.4 Gene expression

2.2.4.1 RNA isolation and cDNA synthesis

Total RNA was extracted from up to 100 mg of frozen root and shoot samples using RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions, and immediately stored at -20^{O}C. Genomic DNA contamination was prevented by using an RNase-Free DNase Set (Qiagen, Germany). The concentration and purity of the total RNA was assessed using a Nanodrop™ spectrophotometer, and by electrophoresis on 1% (w v^{-1}) agarose gel. Approximately 1 µg of total RNA was converted to cDNA through reverse transcription in a 20 µl reaction with 50 U Expand Reverse Transcriptase (Roche, Mannheim, Germany), 50 pmol oligo (dT) primers and 100 pmol random hexamer (pdN6) primers. The final reaction mix was incubated at room temperature for 10 min, then at 42°C for 60 min, and then at 70°C for 15 min to deactivate the enzyme. The cDNA was diluted 10-fold with nanopure water and stored at -20°C.

2.2.4.2 Target gene isolation and sequence analysis

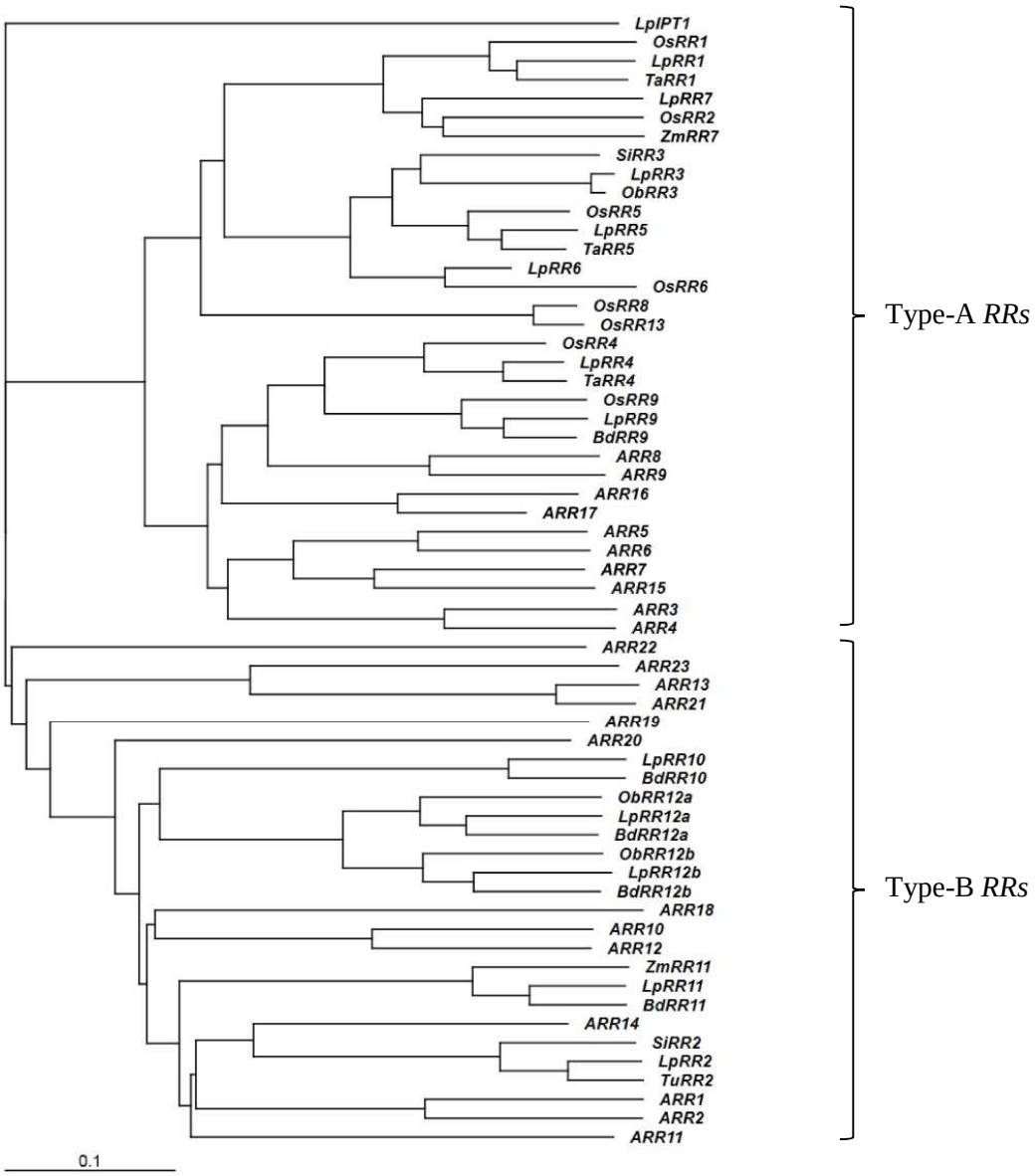
Sequences of candidate *RR* gene family members in perennial ryegrass were determined by Professor Jiancheng Song (School of Life Sciences, Yantai University, Yantai, China, and previously School of Biological Sciences, University of Canterbury, Christchurch, New Zealand) through BLAST searching the NCBI database and a RNA-Seq transcriptome database containing 169 862 assembled sequence contigs of 595 bp in average length generated using an Illumina HiSeq2000 genome analyser at Macrogen Ltd, Korea. Perennial ryegrass cv. Nui was used for transcriptome data generation. A pool of combined RNA samples extracted from multiple developmental stages of leaves, flower spikes, and seeds, was used to construct the cDNA library. All available sequences of *RR* gene families in the GenBank database (Table 2.2) in perennial ryegrass and closely related species including *Brachypodium distachyon*, *Oryza sativa*, *O. brachyantha*, *Zea mays*, *Triticum aestivum*, *Festuca pratensis*, *Hordeum vulgare*, *Setaria italica*, and *Arabidopsis thaliana* were used as query sequences to BLAST search the perennial ryegrass transcriptome database using prfectBLAST 2.0 software. The GenBank accession numbers for the nucleotide sequences are listed in Table 2.2.

Table 2.2. GenBank accession numbers.

Gene family member	Accession numbers
LpRR1	BankIt1870816 LpRR1 KU136270
LpRR2	BankIt1870816 LpRR2 KU136271
LpRR3	BankIt1870816 LpRR3 KU136272
LpRR6	BankIt1870816 LpRR6 KU136273
LpRR9	BankIt1870816 LpRR9 KU136274
LpRR10	BankIt1870816 LpRR10 KU136275
LpRR12a	BankIt1870816 LpRR12a KU136276
LpRR12b	BankIt1870816 LpRR12b KU136277

The putative sequences were verified via BLAST searching the GenBank database and via multiple sequence alignment with representative orthologue sequences in closely related species.

Neighbor-joining (NJ) phylogenetic trees of the newly identified sequences and their orthologues (listed above) were created using ClustalX2 software with 1000 bootstrap replicates. The phylogenetic tree was visualised with TreeView X software. The tree was rooted with an out group sequence from *L. perenne* (Fig. 2.1).



Type-A: LpRR1=Lp7495, LpRR3=Lp2638, LpRR4=Lp7519, LpRR5=Lp3371, LpRR6=Lp3900, LpRR7=Lp8806, LpRR9=Lp7049,

Type-B: LpRR2=Lp6625, LpRR10=Lp7296, LpRR11=Lp4962=Lp0396, LpRR12a=Lp7656=Lp2183=Lp2138, LpRR12b=Lp7466

Figure 2.1. Phylogeny of *LpRR* gene family. Neighbor-joining (NJ) phylogenetic trees of the newly identified sequences and their orthologues (listed above) were created using ClustalX2 software with 1000 bootstrap replicates. Phylogenetic tree was visualised with TreeView X software. The tree was rooted with an out group sequence of *IPT1*, in *Lolium perenne*. *Brachypodium distachyon* Bd, *Lolium perenn*e Lp, *Oryza Brachyantha* Ob, *Oryza sativa* Os, *Seteria italica* Si, *Triticum aestivum* Ta, *Triticum urartu* Tu, Zea mays Zm.

2.2.4.3 Quantitative reverse transcription polymerase chain reaction

Samples preparation and gene expression analysis were completed at the University of Canterbury (Christchurch, New Zealand). Quantitative RT-PCR (RT-qPCR) was used to measure relative gene expression of the individual family members. Specific PCR primers were designed for each *LpRR* gene family member using Primer Premier 6.20 (Table 2.3).

Table 2.3. *LpRR* primer sequences. F, forward; R, reverse

Primers	**Sequence**
***LpRR1*F**	GGAGCAGGCCATCGACATGGTG
***LpRR1*R**	CAGGAGGCGTCCTTCGGTTTCA
***LpRR2*F**	CCAGTTCGTCTAGCTTTCAGAGTTCC
***LpRR2*R**	GCCTTCACATCTGTCCACTAAATCCG
***LpRR3*F**	ATCGTCGGAGCTGAAGCAGATTC
***LpRR3*R**	CTGACAGGCTTGAGCAGGAACTC
***LpRR6*F**	CGGGATGACCGGCTACGAGC
***LpRR6*R**	GCACGTTCTCGGAGGACATGATG
***LpRR9*F**	AGAATCAATAGGTGCCTGGAGGAAGG
***LpRR9*R**	TGTGCCTTGGTCTGCTTGTCTTG
***LpRR10*F**	CCAACCAGCACCCATTCTCAGTC
***LpRR10*R**	GCCGCCAGTGATACACCATTTGA
***LpRR12a-2138*F**	AGACGGCTAAGTGTTGTGGCATCA
***LpRR12a-2138*R**	GGATTGAAAGATGGAATACCAGCAGAAGAG
***LpRR12a-2183*F**	GCAGGATTCTAGTATATCCCAGCAGTGT
***LpRR12a-2183*R**	TGCCAGAAGAACGAGTTCCACATTTG
***LpRR12b*F**	GTTCCACAAGCGAAGATTGATTTCCTC
***LpRR12b*R**	AAGCCCCGAGCGAGTAGAAGTC

In most cases, four primer pairs were designed and the best pair was chosen for gene expression analysis after PCR testing. A volume of 20 µl was used for all RT-qPCR reactions in a Rotor-Gene Q system real-time PCR instrument (Qiagen Hilden, Germany), using home-made SYBR Green master mix. PCR products for each target sequence were Sanger sequenced to confirm homology to genes already identified in various gene databases (e.g., NCBI). RT-qPCR systems were then optimised. Two reference genes, elongation factor 1 alpha (*EF*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as internal controls. Three technical replicates were carried out for one sample set of five pooled plants. For each cDNA sample, the Ct values of each target gene were corrected using a correction factor calculated as described previously (Song et al. 2012). Namely, for each reference gene, a correction factor (CF) was determined for each cDNA sample by dividing the Ct number of the cDNA sample by the average Ct number of all cDNA samples used in the experiment. The values of the three technical replicates were averages in order to obtain the CF of each biological replicate for each reference genes. A final CF was calculated for each biological replicate by averaging the CF of the two reference genes. This final CF was used to correct the Ct number of each target gene for each cDNA sample before statistical analysis. Therefore, the expression values relative to *EF* and *GAPDH* were calculated based on the methods of Pfaffl (2001) and modified as described in Song et al. (2012)(Appendix Table 2.1).

2.2.5 Statistical analysis

The variables were subject to a two-way ANOVA with tissue (root, shoot) and time (0 = pre-treatment, and 1, 3, 7 for one, three, and seven days after N addition respectively) as the factors. Significant effects were defined by Dunnett's multiple comparisons test. Results of an ANOVA were considered statistically significant when $p < 0.05$. Relative abundance in C units in DP2, DP3-6, and HMW WSCs, and in amino acids was considered significantly different relative to pre-treatment (d0). Similarly, the statistical significance in the cytokinin contents in the KCl- or KNO_3 -treated plants at d1 and d7 was estimated relative to d0.

2. 3 Results

2.3.1 Physiological and growth responses to added nitrogen

In order to investigate the early response of KNO_3 addition on N-deficient plants, total C and N content were measured in plants grown initially in unfertilised soil, and then exposed to 5 mM KNO_3 or KCl (control) for one, three, or seven days. Shoot-N content was higher (Fig. 2.2A) and C:N ratio was lower than in the root (Fig. 2.2B).

The N content in N-treated roots increased rapidly within d1 of N treatment, and was associated with a decrease in the C:N ratio, whereas the shoot dynamics changed more gradually over the course of the experiment. In order to determine the N use efficiency of perennial ryegrass plants in hydroponics, leaf and tiller numbers were measured and had increased significantly within six days following KNO_3 addition (Fig. 2.2C, D).

2.3.2 Water-soluble carbohydrates

In order to understand the dynamics of metabolic changes over time in response to N addition, a metabolic profiling was conducted. N-deficient plants at day zero had accumulated WSCs in above- and below-ground tissues with relatively greater storage abundance of WSCs in the shoot (Fig. 2.3A, B).

When calculated per unit of C, disaccharides were the most abundant WSCs regardless of the tissue type (Fig. 2.3A, B). The WSCs with degree of polymerisation (DP) from three to six, referred to here as low molecular weight (LMW) WSCs, were less abundant relative to the disaccharides. By contrast, the relative contribution in terms of C units from the high molecular weight (HMW) WSCs increased from DP7 to DP14 and then decreased for the WSCs of higher DP (Fig. 2.3A, B). Shoot and root tissues presented different profiles in the relative C contribution from the WSCs. Before N addition, C units present in DP2 had accumulated slightly more in the shoot (Fig. 2.3C). There were no statistically significant differences in the abundance of other LMW WSCs (DP3-6) between tissue types (Fig. 2.3D).

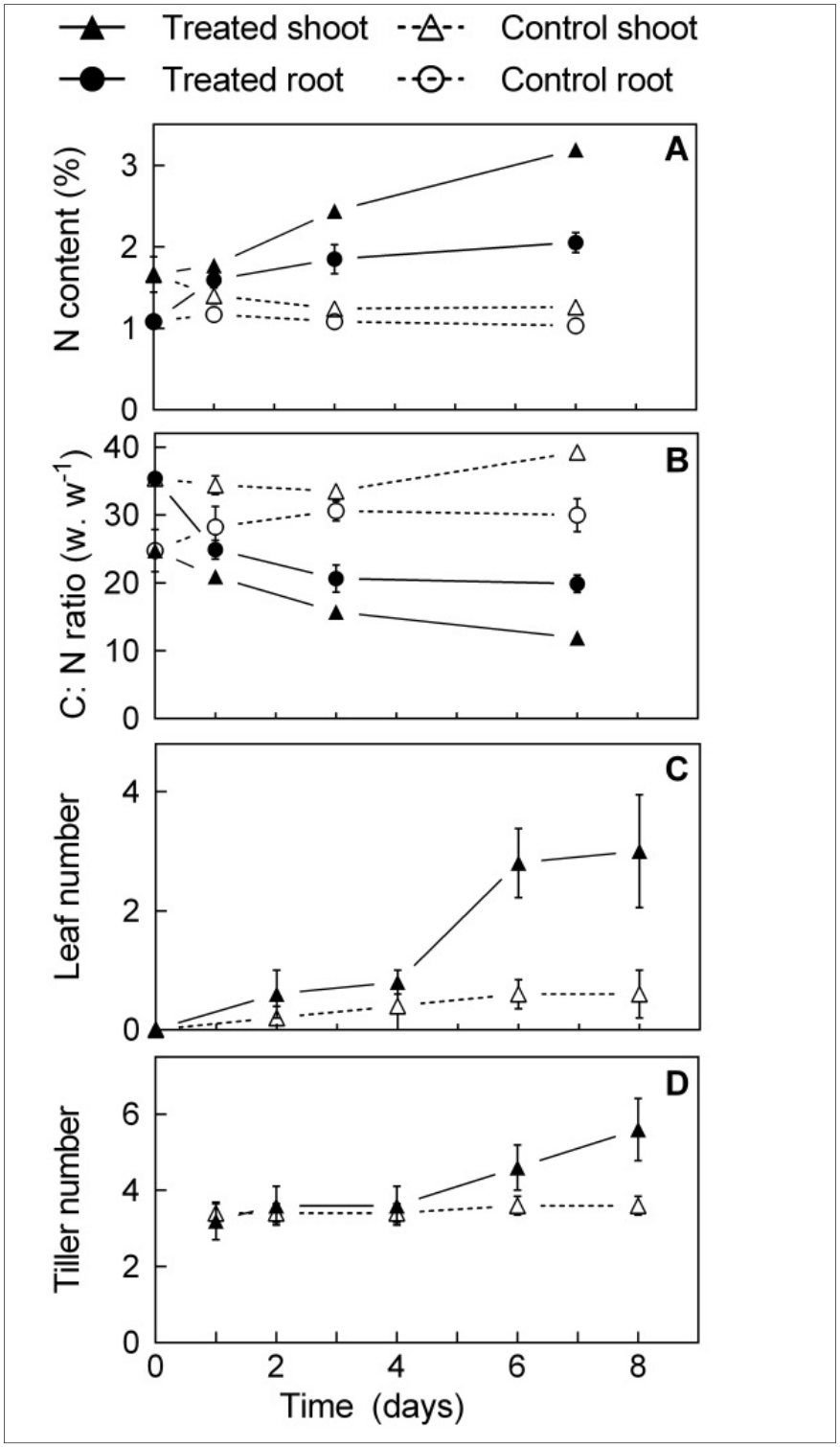


Figure 2.2. Effect of NO_3^- addition on total N content (A), C:N ratio (B), leaf appearance (C), and tiller number (D) of perennial ryegrass plants. Nitrogen starved plants were treated with 5 mM KCl (Control) or 5 mM KNO_3 (Treated) on day zero. Data are means ± SE, n = 4 pools of five plants for N content and C:N ratio; n= 5 for leaf number and tiller number.

However, more substantial differences were observed between root and shoot for the HMW WSCs (Fig. 2.3E). As a general trend, the higher the DP, the more likely it was to be accumulated in the shoot rather than in the root (Fig. 2.3F). HMW WSCs were three times more prevalent in the shoot than in the root at pre-treatment (d0). In particular, DP31 was the most prevalent WSC in the shoot and provided seven times more C than did DP31 in the root.

A decrease in all WSCs was observed in response to N addition (Fig. 2.3). However, the dynamics of responses differed between LMW and HMW WSCs, and tissue types. In the root, the breakdown of HMW WSCs was observed mainly between d1 and d3, and subsequently stabilised (Fig. 2.3B), whereas in the shoot the total HMW WSCs decreased gradually after KNO_3 addition (Fig. 2.3E). Although LMW WSCs in the root were significantly reduced by d1, changes in the relative abundance in C units of LMW WSCs in the shoot had become significantly reduced only by d7.

2.3.3 Metabolites associated with glycolysis, tricarboxylic acid cycle, and amino acids

The TCA cycle intermediates, citric acid, fumaric acid, and malic acid, decreased significantly and rapidly within a day after N supply in the root (Fig. 2.4E) but fumaric acid and malic acid did not change significantly over time in the shoot. The major N-carrier amino acids Gln, Asp, and Asn accumulated after N-treatment with similar patterns between tissues and showed comparatively higher relative abundance in the shoot than in the root (Fig. 2.4D, F). Glutamine accumulated within the first day, and subsequently decreased in the shoot. The increase in Asp and Gln represented the strongest difference in relative abundance (difference of peak intensity) observed after KNO_3 addition. Aspartic acid and Asn were characterised by a notably longer accumulation, reaching their highest intensity by d3, followed by a plateau (Fig. 2.4D). This pattern was also observed for Ile, Leu, and Ala in the shoot (Fig. 2.4D, C), and was distinct from their patterns in the root: root Ile and Leu did not change significantly, and root Ala accumulated during d1, and subsequently decreased. More complex variations were recorded for Gly, Thr, and Val, for which the trends contrasted over time and between tissues (Fig. 2.4A, D, C). Among the other N-rich amino acids, Glu did not change significantly over the course of the experiment, and His and Arg trends remained unknown due to a lack of annotations (Fig. 2.4F). A rapid decrease in the aromatic amino acids Trp, and root Tyr and Phe, was observed following N addition (Fig. 2.4B)

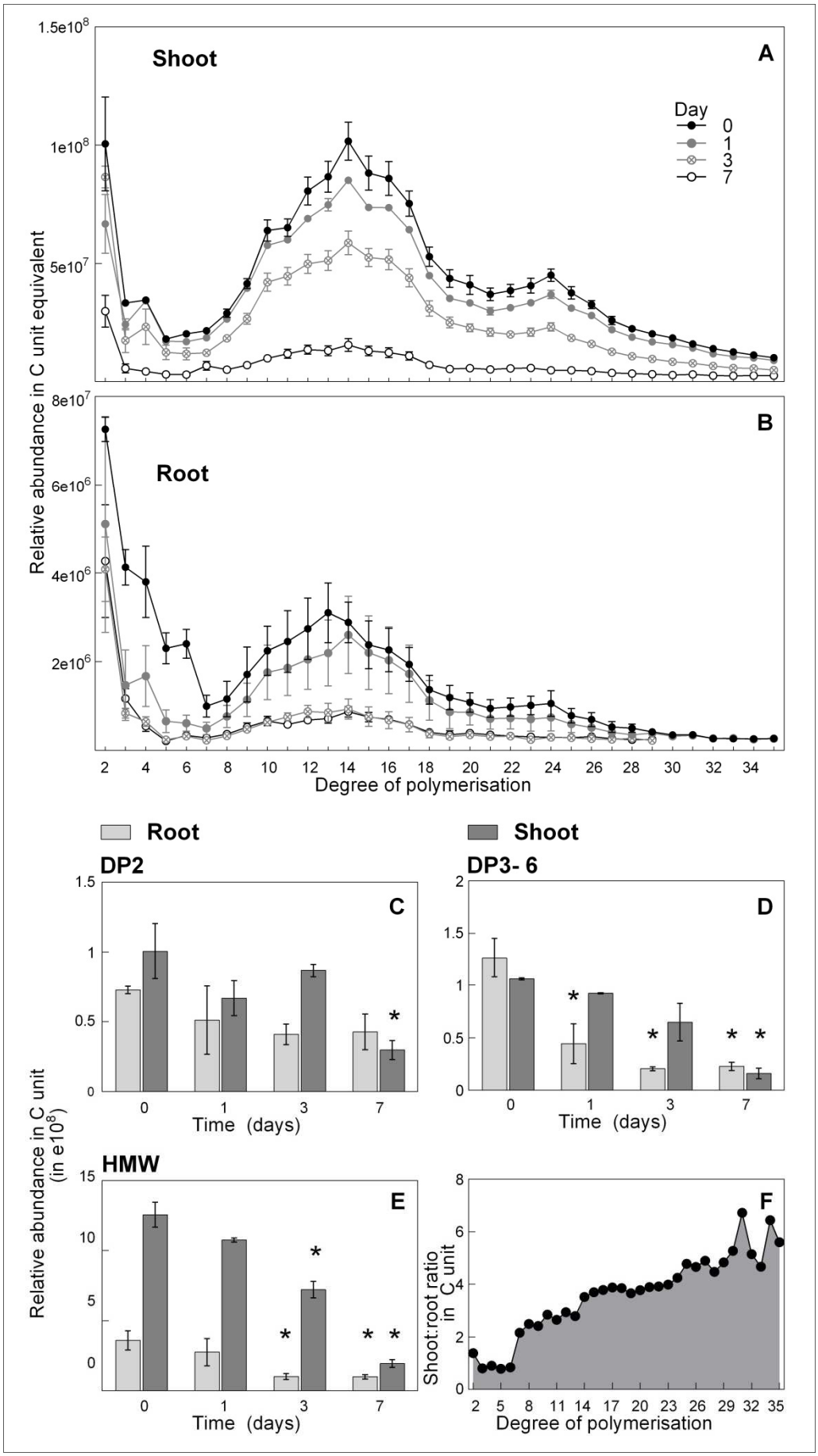

Shoot
A
Day
0
1
3
7
Relative abundance in C unit equivalent
1.5e10^8
1e10^8
5e10^7
Root
B
8e10^7
6e10^6
4e10^6
2e10^6
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Degree of polymerisation
Root
Shoot
DP2
C
DP3- 6
D
Time (days)
HMW
E
Relative abundance in C unit (in e10^8)
F
Shoot:root ratio in C unit
2 5 8 11 14 17 20 23 26 29 32 35
Degree of polymerisation

Figure 2.3. Relative abundance in C units of water-soluble carbohydrates (WSCs) measured over the seven-day period following 5 mM KNO_3 addition to the hydroponic solution of N-deficient perennial ryegrass plants. Relative abundance in C units was calculated by multiplying peak intensity degree of polymerisation (DP). WSC profiling in shoots (A) and roots (B); relative abundance of WSC of degree of polymerisation two (C), three to six (D), and high molecular weight (E); and ratio of shoot to root relative abundance in fructans at d0 (F). 0 = pre-treatment, and 1, 3, 7 for one, three and seven days after N addition respectively. Data are means ± SE, n = 3, where each replicate is a pool of five plants. Asterisks indicate a significant difference from the pre-treatment (0) in root and shoot tissue.

2.3.4 Cytokinin content and expression of cytokinin response regulator genes

As a general trend, N addition resulted in a significant augmentation of the cytokinin content relative to d0 (Fig. 2.5; see Appendix 2.3 for the complete data set).
In particular, the *trans*-zeatin-types were characterised by a highly significant peak at d1. Along with an increase in *t*ZRMP, iPRMP accumulated in response to N, although the response of iPRMP appeared similar to that in both the KNO_3- and KCl-treatment of the root at d1. An accumulation of *t*ZR and iPR occurred following N addition in both tissues. The iPR increase was also associated with a response to KCl treatment in the shoot.

The N-induction of the active free-bases was stronger in the shoot compared to the root. Indeed, the quantity of *t*Z and iP was *ca.* twice as great in the shoot as in the root following N-treatment (Fig. 2.5A). During the course of the experiment, *t*Z and iP levels remained significantly higher relative to pre-treatment up to d7 in the shoot, but only up to d1 in the root. *Trans*-Z accumulation was specific to N-treatment (Fig. 2.5A). In contrast, accumulation of iP-types was also observed in response to KCl (Fig. 2.5A, B). *Trans*-Z-glucosides increased significantly in response to N-treatment: by d1 for *t*ZOG in the shoot; by d7 for *t*ZOG, *t*ZROG, *t*Z7G and *t*Z9G in the shoot and *t*ZROG, and *t*Z9G in the roots (Fig. 2.5A). iP9G increased in response to KCl at d1 in the roots (Fig. 2.5B). The DZ-type cytokinins were all below 3 pmol g DW^{-1} in perennial ryegrass, regardless of the treatment type, tissue type, and time point (Fig. 2.5 and Appendix 2.3).

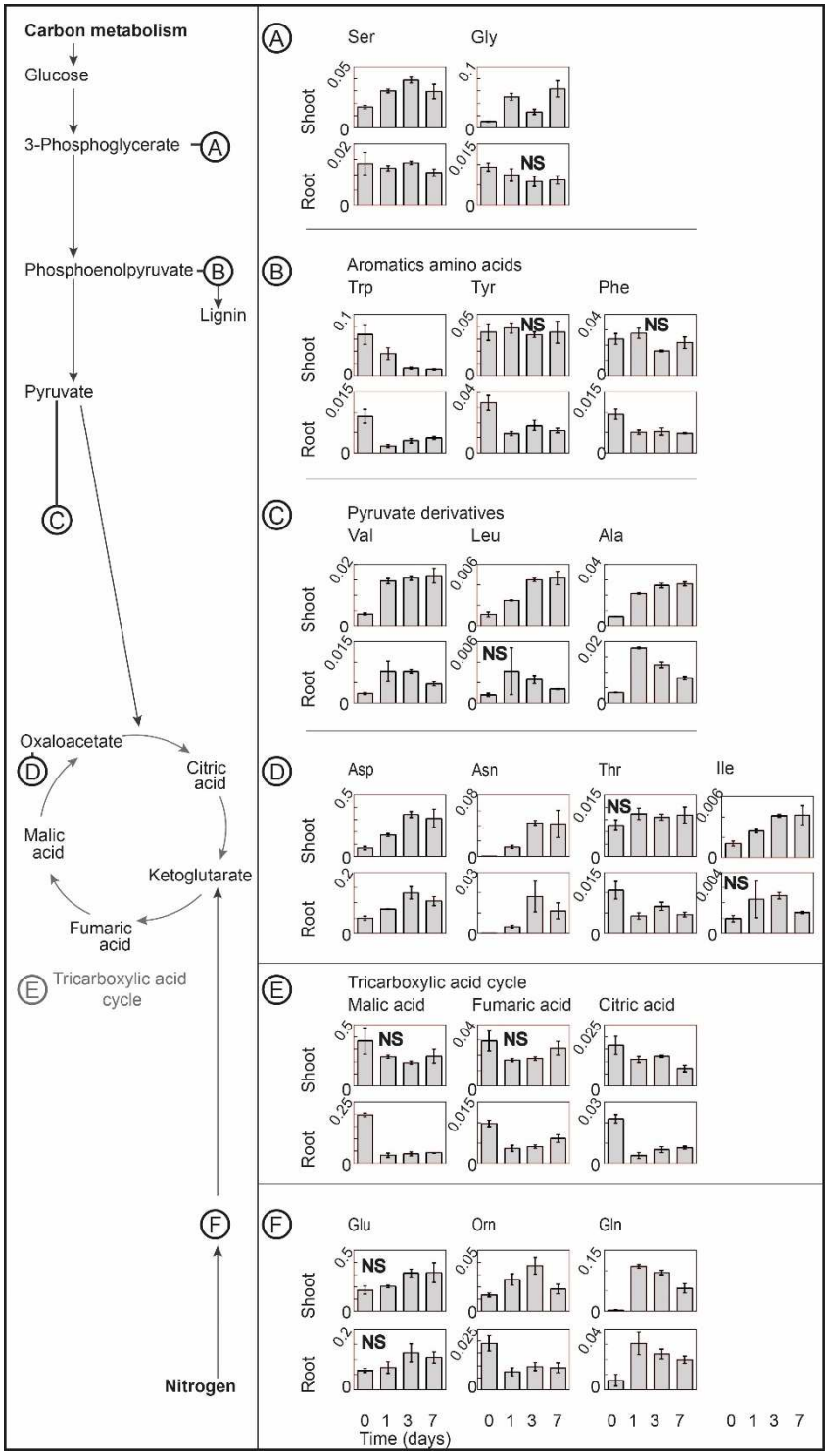


Figure 2.4. Mapping of metabolite changes in values representing relative abundance alongside known pathways immediately before and at one, three and seven days following 5 mM KNO_3 treatment of perennial ryegrass plants in hydroponic solution. 0 = pre-treatment, and 1, 3, 7 for one, three and seven days after N addition respectively. NS: not statistically significant compared to pre-treatment within the same tissue; n = 3 where each replicate is a pool of five plants.

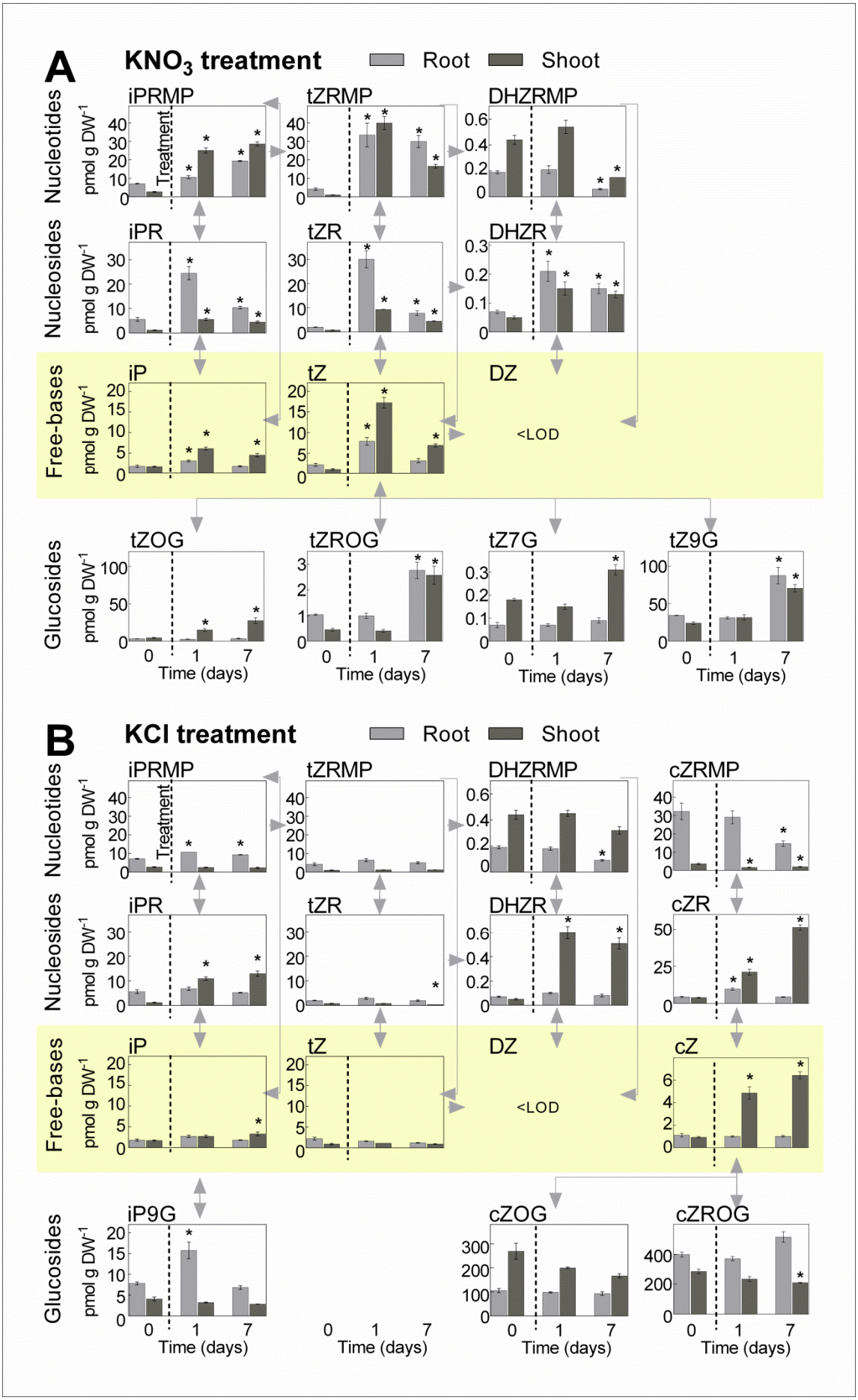

A
KNO3 treatment
Root
Shoot
Nucleotides
Nucleosides
Free-bases
Glucosides
iPRMP
tZRMP
DHZRMP
iPR
tZR
DHZR
iP
tZ
DZ
<LOD
tZOG
tZROG
tZ7G
tZ9G
pmol g DW-1
Treatment
Time (days)
B
KCl treatment
Root
Shoot
cZRMP
cZR
cZ
iP9G
cZOG
cZROG

Figure 2.5. Cytokinin content of perennial ryegrass roots (light grey) and shoots (dark grey). 0 = pre-treatment, and 1, 7 for respectively one and seven days after 5 mM of KNO_3 treatment (A) or KCl treatment (B). Data are means ± SE, n= 3 where each replicate is a pool of five plants. Dihydrozeatin (DZ) content was below 1 pmol g DW^{-1} and other non-significantly changing cytokinin forms are not shown. The complete dataset is in Appendix 2.3.

In contrast to the *trans*-cytokinins and iP-type cytokinins, none of the *cis*-forms increased following N-treatment. The majority of the changes in the *cis*-forms occurred following the KCl-treatment (Fig. 2.5B). In particular, *c*ZR and *c*Z levels increased in the shoots, with a smaller increase of *c*ZR at d1 in the roots. The *cis*-glucosides, *c*ZOG and *c*ZROG, were detected at the greatest level of all the cytokinins at d0, noting that *c*ZOG was detected at its greatest level in the shoot tissue, whereas *c*ZROG was at its greatest level in the roots. KCl-treatment induced a significant decrease in *c*ZROG at d7 in the shoot, and a similar but not significant decrease in *c*ZOG. In addition, decreases in *c*ZRMP were detected following both KCl- and KNO_3-treatments.

In order to decipher whether the variation in cytokinin activated a cytokinin signalling pathway, the expression of putative cytokinin response regulators was recorded using RT-qPCR analysis for relative quantification (Fig. 2.6).

All of the up-regulated *LpRR* family members were Type-A *LpRRs*. However, *LpRR3* was down-regulated in both shoots and roots. All Type-B *LpRRs* were down-regulated following KNO_3 addition. The only *LpRR* family member remaining within two-fold changes before and after KNO_3-treatment, in both shoot and root tissues, was Type-A *LpRR6*. Overall, the root and shoot profiles contrasted mainly by the responses of the various *LpRR* family members and their associated timing of expression. In the root, four of the eight *LpRR* members identified remained within two-fold changes following N-addition, whereas a shoot-specific down-regulation was identified for *LpRR2*, *LpRR12a*, and *LpRR12b*. Therefore, most of the *LpRRs* were down-regulated in the shoot, especially at d1, which contrasted with fewer *LpRR* members regulated in the root.

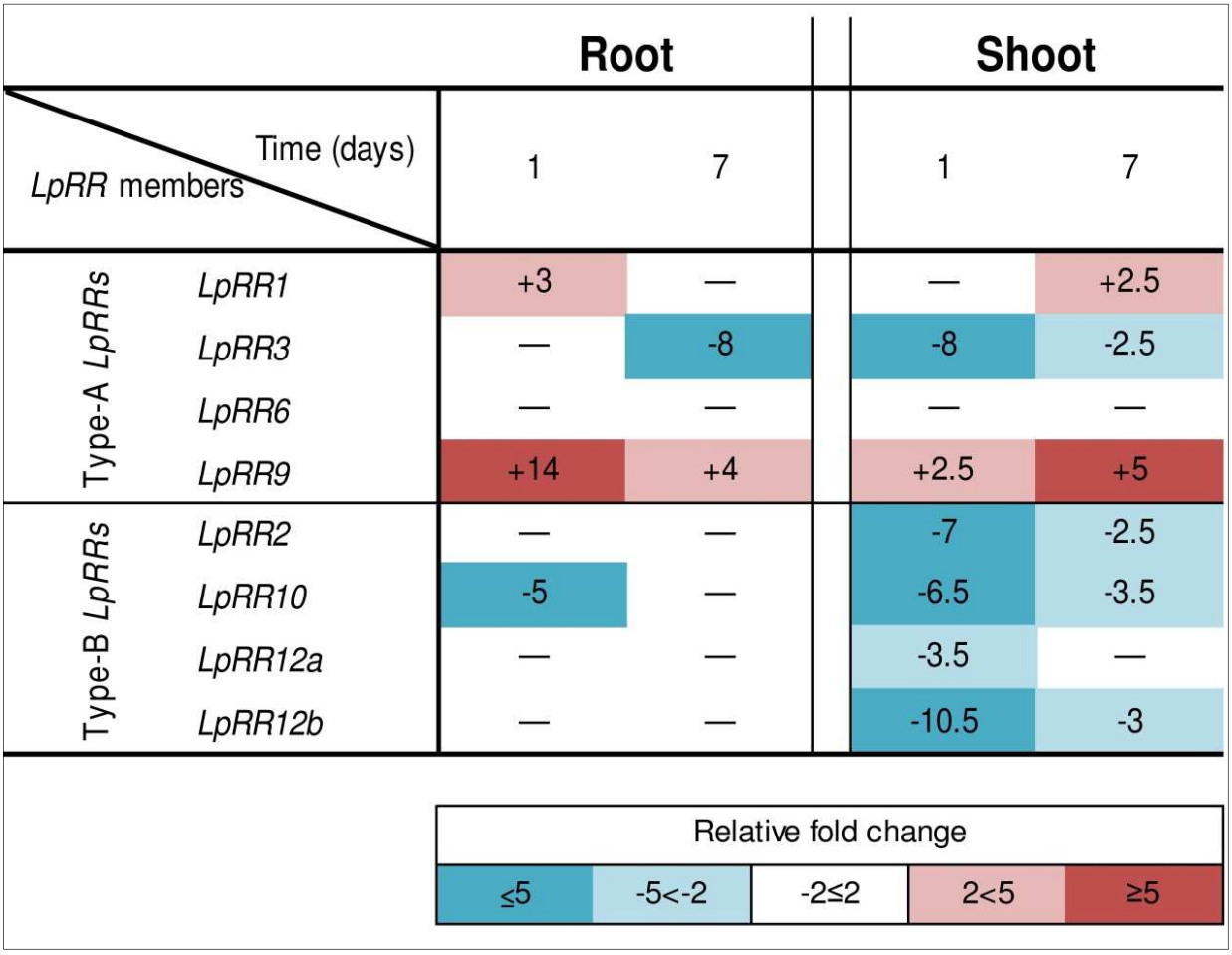


Figure 2.6. Relative expression of cytokinin response regulator (RR) gene family members in root and shoot tissues of perennial ryegrass. Positive (+) or negative (-) values were fold changes relative to the expression level immediately before treatment calculated using reference genes *GAPDH* and *EF* as internal controls. Numbers indicated in each cell correspond to relative fold change in expression. 0 = pre-treatment, and 1, 7 for one and seven days after N addition respectively. Each value represents the mean of three technical replicates using a cDNA from a pool of five plants as the RT-qPCR template.

2. 4 Discussion

2.4.1 High molecular weight water-soluble carbohydrates supply carbon for nitrogen metabolism

Consistent with Louahlia et al. (2008), N-deficient perennial ryegrass plants accumulated WSCs, and showed a limited assimilation of NO_3^-, with reduced levels of some free amino acids particularly at d0 for the nitrogenous amino acids Gln and Asn, and the aliphatic amino acids Leu, Ile, Val, and Ala (Fig. 2.4). When N was resupplied, the opposite trends were recorded (Figs. 2.2 and 2.4).

The total N content was greater in shoot than in root tissue, irrespective of the N-treatment (Fig. 2.2A), indicating that the shoots of perennial ryegrass are the primary organ for N storage and utilisation. Additionally, as most of the HMW WSCs were located in the shoots rather than in the roots (Fig. 2.3A, B, F), this suggested that the shoots are also the main organ for C storage in young perennial ryegrass plants. Under the experimental conditions, N-treatment was associated with a shift of amino acids towards a greater accumulation in the shoot rather than in the root (Fig. 2.4), suggesting that the amino acids could be synthesised or accumulated preferentially in the above-ground parts of perennial ryegrass to support shoot growth in response to N addition. These results are consistent with the study of Bowman and Paul (1988) who showed, following a NO_3^- treatment on N-limited perennial ryegrass plants, that an initial phase of NO_3^- reduction in the root during the first 12 h following NO_3^- treatment was followed in time by a greater NO_3^- reduction in the shoot (Bowman and Paul 1988).

Three main phases of responses were identified following NO_3^--treatment to N-depleted perennial ryegrass plants. The first phase took place within one day and highlighted the importance of the root in the initial N-response (Fig. 2.2A). It was characterised by a rapid remobilisation of C from root LMW WSCs (Fig. 2.3B, D), combined with a significant increase in root N content, and a rapid biosynthesis of free amino acids (Fig. 2.4). Initially, the HMW WSCs may have been maintaining the pool of LMW WSCs, especially in the shoot. The second phase occurred between d1 and d3. The shoot became the main tissue for N accumulation (Fig. 2.2A) and the main source of C from WSCs by remobilisation of a large pool of HMW WSCs that had accumulated in the shoot under N-limiting conditions

(Fig. 2.3A, E). In contrast, root N content had stabilised by d3 (Fig. 2.2A) and the root WSC reserves had almost become exhausted (Fig. 2.3B, D). Most amino acids stabilised by d3, with a higher abundance noted in the shoot (Fig. 2.4). The third phase was in place by d7. By that time, the shoot N content had continued to increase (Fig. 2.2A). Interestingly, the shoot HMW WSCs continued to steadily breakdown whereas the root reserves were exhausted (Fig. 2.3B, E). Some of the amino acids that initially increased in response to N addition in the root decreased at d7 (Fig. 2.4), suggesting that the peak of amino acid production was over by this time. The data indicate a decline in NO_3^- assimilation within one week of N fertilisation in the root, and to a lesser extent in the shoot.

Interestingly, this trend was only observed for the amino acids Gln and Orn in the shoot. Nitrate assimilation into amino acids is initiated by reduction of NO_3^- to nitrite by the enzyme nitrate reductase. Nitrite is subsequently converted to NH_4^+ by the nitrite reductase enzyme located in the root plastids and shoot chloroplasts (Masclaux-Daubresse et al. 2010). Glutamine and Glu are considered to be the primary products of NH_4^+ assimilation with the GS/GOGAT enzymes (glutamine synthetase and glutamate synthase) acting as a catalyst in a cycle in which Glu is both a substrate and end product (see section 1.2.3). Considering that Gln is a precursor to all other amino acids, the stable relative abundance of all the amino acids, with the exception of Gln and Orn, in the shoot by d7 might be explained by either a steady rate of biosynthesis that could potentially affect Gln mobilisation, or an amino acid import from the root to the shoot or, possibly, a lower depletion of amino acids in the shoot by d7. Notably, shoot DP2 had only decreased significantly by d7 (Fig. 2.3C). However, caution must be taken when estimating the C fluxes from measurements of steady-state level of DP2, because of issues of stability and compartmentalisation that can lead to misleading biological interpretation (Fernie et al. 2005).

2.4.2 Tricarboxylic acid cycle intermediates decrease in response to nitrogen

Treatment with KNO_3 was associated with a decrease in the TCA cycle intermediates citric acid, fumaric acid, and malic acid in the root (Fig. 2.4E). This is opposite to the results from a metabolomics analysis of mature perennial ryegrass plants (Rasmussen et al. 2008). They showed that malate, succinate, and citrate were all higher for plants grown at steady-state high N supply compared to plants with reduced N supply. This might represent a developmental response, as the results obtained in this study are consistent with increased

levels, especially in the roots, of citric acid, fumaric acid, and malic acid recorded in *A. thaliana* plants that were N-starved for 10 days (Krapp et al. 2011). Previously, Tschoep et al. (2009) reported that *A. thaliana* plants under a mild but sustained N-limitation showed increasing fumaric acid content. In addition, *A. thaliana* leaves have often been reported to have very high levels of fumaric acid, sometimes exceeding those of starch and soluble sugars (Chia et al. 2000). Fumaric acid has thus been hypothesised to act as a temporary C sink for photosynthate which can be remobilised to yield energy and C (Chia et al. 2000), and may have a function in C partitioning between different plant parts (Tschoep et al. 2009). *A. thaliana* knockout mutants of a cytosolic fumarase suggested an important role of the fumaric acid/malic acid homeostasis in maintaining C metabolism, as well as rapid N assimilation and growth on high N (Pracharoenwattana et al. 2010). Under the experimental conditions of this experiment, fumaric acid and malic acid decreased statistically significantly in the root and did not decrease significantly in the shoot of perennial ryegrass in response to KNO_3 treatment, which suggests that fumaric acid and, potentially, malic acid could act as a flexible alternative C store influenced by the N status in a tissue specific manner.

2.4.3 Cytokinins respond to both the presence and absence of nitrogen

Plants possess intricate regulatory machinery able to coordinate N use with C metabolism, and thus the control of the C/N ratio (see section 1.4) (Nunes-Nesi et al. 2010). Changes in cytokinin occurred in response to the addition of both KNO_3 and KCl (Fig. 2.5). According to Takei et al. (2004) changes in cytokinin levels occurred within 24 h following the resupply of N to N-starved maize plants. In agreement with Takei et al. (2004), the cytokinin measurements show extensive accumulation and also metabolism of cytokinin, both in roots and shoots, at 24 h following N supply. Even within 24 h, *t*ZOG had increased in the shoots and, over 7d, both storage (*t*ZOG, *t*ZROG, *c*ZROG) and inactivated forms (*t*Z7G, *t*Z9G) had accumulated, indicating that cytokinin homeostatic mechanisms were activated rapidly in shoots.

Two maize Type-A cytokinin *RRs*, that were up-regulated following addition of NO_3^-, were initially described as N-responsive genes but were subsequently shown to be activated directly by cytokinin and cytokinin-mediated N responses, and not by N itself (see sections 1.4.2 and 1.4.3) (Yamada et al. 2001). The data indicate that, following N addition to perennial ryegrass, the up-regulated *LpRRs* were all Type-A *LpRRs* (Fig. 2.6). However,

within 24 h of N addition, all the Type-B *LpRRs* in the shoot were down-regulated, indicative of a feedback mechanism dampening the NO_3^--induced cytokinin responses. Clearly, cytokinin biosynthesis, metabolism and signalling is associated with the transition of perennial ryegrass from N-starved to N-replete.

Changes in cytokinin content were observed in response to KCl, particularly the *c*Z- and the iP-type cytokinins (Fig. 2.5B). As found in other members of the Poaceae (Gajdošová et al. 2011), relatively high levels of cZ-type cytokinins were detected in perennial ryegrass. In particular, the storage forms *c*ZOG and *c*ZROG were detected at one order of magnitude greater than any other cytokinin at d0, at which time the plants had been growing on unfertilised soil for 10 weeks. The *c*Z- and iP-type cytokinins are normal constituents of certain tRNAs (see section 1.4.2) (Sakakibara 2006). Schäfer et al. (2015) suggested that various stress conditions induce tRNA turnover pathways. They noted that *c*Z-containing tRNA can be formed from hydroxylation of the iP-containing tRNA, and the action of an unknown enzyme on these tRNAs could release *c*Z/*c*ZR forms and iPR forms, respectively (Schäfer et al. 2015). Consequently, enhanced tRNA turnover could explain the increase in both *c*Z- and iP-type cytokinins in response to KCl-treatment in this experiment. Interestingly, wheat plants exposed to ammonia as sole N source also showed enhanced levels of both *c*Z- and iP-type cytokinins (Garnica et al. 2010). The data obtained in this study are consistent with the hypothesis presented by Gajdošová et al. (2011) that *c*Z and/or its derivatives might maintain minimal levels of cytokinin under growth-limiting conditions necessary for plant survival and subsequent recovery (Gajdošová et al. 2011).

2. 5 Conclusion

In conclusion, the results presented in this chapter provide a spatiotemporal characterisation of amino acids, tricarboxylic acid intermediates, and WSCs, as well as cytokinin biosynthesis, metabolism, and signalling of N-limited young perennial ryegrass plants following KNO_3 treatment. An initial remobilisation of LMW WSCs was identified within a day of N treatment, followed in time by a remobilisation of a larger pool of HMW WSCs in the shoot. This was associated with a greater accumulation of N and of amino acids in the shoot. Variations in the cytokinin content and *LpRR* gene expression suggest long-distance root/shoot signalling within a day of KNO_3 treatment, and ongoing input from cytokinin over

the seven days.

The work described in this chapter gave us a picture of N assimilation in intact plants. Grasses are able to tolerate defoliation by herbivores by reallocation of C and N resources to support rapid regrowth of photosynthetic tissue (Ferraro and Oesterheld 2002). In the next chapter, the impact of defoliation will be taken into account to understand the physiological underpinnings and mechanisms controlling a coordinated use of C and N taking place during recovery of perennial ryegrass plants following defoliation (see Chapter 3).

Chapter 3

Coordinated nitrogen and carbon remobilisation for nitrate assimilation in leaf, sheath, and root and associated cytokinin signals during early regrowth of *Lolium perenne*

Contents

Roche J, Turnbull MH, Guo Q, Novák O, Späth J, Gieseg S, Jameson PE and Love J (2016) Coordinated nitrogen and carbon remobilization for nitrate assimilation in leaf, sheath, and root and associated cytokinin signals during early regrowth of *Lolium perenne.* (In review)

3. 1 Introduction

Perennial ryegrass (*Lolium perenne* L.) pasture systems usually receive repeated nitrogen (N) fertiliser application after bovine grazing (Miller et al. 2001). Plant response to N fertiliser treatment is initiated by root N uptake and subsequent N assimilation into organic compounds (see sections 1.1 and 1.2.3 for background). Nitrogen assimilation with carbon (C)-accepting molecules initiates the biosynthesis of amino acids, conversion into more complex N-containing molecules and ultimately results in plant growth response (see sections 1.2.1, 1.2.3, 1.3.2, 1.3.2 and Chapter 2). As shown in Chapter 2, N assimilation in perennial ryegrass plants needs to be closely coordinated with the remobilisation of C from water soluble carbohydrate (WSC) reserves (see sections 1.2.3, 1.3.2, 1.3.3 and Chapter 2). Plant hormones, including the cytokinins, have been implicated as local and long-distance signals regulating nutrient use (see section 1.4, Chapters 2 and 4). Further research is needed to investigate the implication of cytokinin signals in the coordinated use of C and N resources during the early regrowth following defoliation (see sections 1.4.3, 1.5, Chapter 2). Grass species such as *L. perenne* are characterised by vegetative meristems located at the shoot base, which provides protection for regrowth and for plant survival following grazing/defoliation (Langer 1979). Perennial ryegrass response to defoliation is associated with a defoliation-induced increase in C demand and includes regulations of transporter systems for root NO_3^- uptake and subsequent translocation to the shoot (see section 1.3.3)(Louahlia et al. 2008). However, the detailed mechanisms underlying the regulation of N assimilation by the internal C/N balance are yet to be established during the regrowth period of perennial ryegrass plants.

In this study, the effect of contrasting internal C/N balance on the efficiency of NO_3^- assimilation was investigated in response to an increase in C demand, experimentally induced by a defoliation treatment of the perennial ryegrass plants. The interaction between C remobilisation and NO_3^- assimilation on biochemical and physiological traits was investigated by measurement of the spatiotemporal remobilisation of WSCs, amino acid profiling, and growth responses over a period of seven days after defoliation. In order to obtain plants with contrasting internal N content and WSC availability, plants were exposed to either continuous light or short days (8 h light: 16 h dark), and to either high (5 mM) or low (50 µM) NO_3^- as a sole N-source. Half of the plants were defoliated and the leaf tissue (leaf material above the

sheath), sheath (pseudo-stem), and roots were harvested at 8 h, 24 h, and 168 h after cutting. Changes in cytokinin content were measured at 8 h following cutting to determine whether cytokinin can act as an early signal integrating C and N supply and demand, and to determine its involvement in the regulation of NO_3^- assimilation after defoliation.

3. 2 Material and methods

3.2.1 Plant material

Perennial ryegrass (*L. perenne* L. cv. "Grasslands Nui") seeds were grown in individual pots at the University of Canterbury glasshouses (43^{O}31'48" S, 172^{O}37'13" E) for 12 weeks using unfertilised soil treated once with commercial NPK fertiliser to maintain plant growth until plant establishment. Seedlings were subsequently watered for two weeks with N-free Hoagland liquid medium (N-free Hoagland medium, BioWorld, USA) to supply all nutrients in sufficient amounts with the exception of N. To obtain plants with different carbohydrate status and N content, perennial ryegrass plants were then transferred to controlled-environment rooms (University of Canterbury) under either continuous light (irradiance of 57-67 quanta µmol m^{-2} s^{-1}), subsequently referred to as D, or short days (8 h light: 16 h dark, irradiance of 70-98 quanta µmol m^{-2} s^{-1}), subsequently referred to as d, and watered with N-free Hoagland medium modified with either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source for two weeks until the time of defoliation. Half of the plants were defoliated at 4 cm above the crown tissue at time 0 of the experiment and subsequently leaf, sheath, and root tissues were harvested at 8 h, 24 h, and 168 h after cutting, flash frozen in liquid N, and stored at -80^{O}C. Leaf tissue corresponded to the foliar/lamina material located above the sheath. Sheath tissues are here defined as the pseudo-stem tissue located between crown and ligule, including the leaf bases and mature leaf sheaths. Three biological samples were used for subsequent analysis. Each biological replicate was comprised of pooled tissue samples harvested from seven plants. Samples were ground into fine powder under liquid N for further analysis. Measurements of total N content and total C content were obtained by isotope ratio mass spectrometry at the Department of Soil and Physical Sciences, Lincoln University, Christchurch, New Zealand.

3.2.2 Fructan analysis

Tissue samples harvested from three biological replicate were ground under liquid N, freeze-dried at the University of Canterbury (Christchurch, New Zealand). Aliquots were sent to the Swedish University of Agricultural Sciences (Swedish University of Agricultural Sciences KBC, Umeå University, Umeå, Sweden) for WSC analysis. Extraction and analysis of low- (LMW) and high-molecular weight (HMW) WSCs were performed using an Agilent 1290 Infinity LC System (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6550 Accurate-Mass QTOF LC-MS system with a dual Agilent Jet Stream source operating in negative mode and a QTOF mass range selected to 70-1700 mass-to-charge ratio. Oligosaccharides of degree of polymerisation two to 20 were extracted from 25 or 10 mg of freeze-dried ground plant material mixed with 750 µL or 300 µL of water, respectively. Samples were vortexed and placed on a heating block for 15 min at 90°C. The tubes were centrifuged for 10 min at 14000 rpm and the supernatant was transferred to a filter tube and centrifuged for 2 min at 14000 rpm. Extracts were transferred to LC vials and aliquots of 2 µL were injected onto an Acquity UPLC HSS T3 C_{18} column (2.1 x 50 mm, 1.8 µm) combined with a 2.1 mm x 5 mm, 1.8 µm VanGuard pre-column (Waters Corporation, Milford, MA, USA) held at 40°C. The linear gradient elution consisted of 0.1 to 10% of solvent B (0.1% FA, 75/25 ACN/IPA) over 2 min, followed by an increase to 99% solvent B for 5 min, and then stabilised for a further 2 min. Subsequently, solvent B was decreased to 0.1% for 0.3 min, and then the flow rate was increased to 0.8 mL min^{-1} for 0.5 minutes and held for 0.5 minutes. Finally, the flow rate was reduced to 0.5 mL min^{-1} and held for 2 minutes before the next injection. Data was collected in centroid mode with an acquisition rate of 4 scans s^{-1} and 1975 transients per spectrum. Data processing was performed on NetCDF files processed and analysed using in-house scripts in MATLAB 7.14.739 (R2012a) (Mathworks, Natick, MA). Water soluble carbohydrates of LMW were defined as oligosaccharides of degree of polymerisation (DP) from DP3 to DP9 and HMW WSCs corresponded to DP10 to DP20.

3.2.3 Amino acid determination

Samples preparation and amino acid determination were completed at the University of Canterbury. Free amino acids were derivatised with Waters AccQ-Tag (Millipore), separated by HPLC, and quantified by fluorescence detection (Excitation, 250 nm; Emission, 395 nm)

as previously described (Reverter et al. 1997). Samples were processed as follow: 300 µL of milliQ Ultrapure water was added to approximatively 25 mg DW of freeze-dried ground plant material. Samples were vortexed for 1 min, centrifuged for 10 min at 15 000 g. The extraction was performed in a new tube by adding 250 µl of chloroform and 900 µl of methanol to 50 µL of supernatant. Samples were vortexed again for 30 sec and centrifuged for 5 min at 13 000 g. The supernatants (50 µL) were transferred to a new tube, freeze-dried for 30 min, reconstituted in 20 µL of 20 mM HCl, and derivatised with 20 µL of the δ-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) following the Waters AccQTaq Chemistry Package Instruction Manual (WAT05287 Rev. 1, Waters, Milford MA, USA). Derivatised amino acids were separated by reversed phase HPLC on an AccQ-Tag 60A column, 4 µm (Waters, NZ; 150 x 3.9 mm), and detected with a fluorescence detector (RF-10Ax, Shimadzu; Ex:250nm, Em:395nm). Amino acids detected and quantified were: Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser,Thr, Tyr, Val. Co-eluted peaks for His and Gln, and for Ser and Asn were subsequently referred to as His + Gln and Ser + Asn respectively. Amino acids were identified and quantified by comparison with Waters amino acid hydrolysate standard. Glutamine and Asn standards were obtained separately (Sigma chemical Co., St Louis, USA).

3.2.4 Endogenous cytokinin quantification

As indicated in Chapter 2, plant samples were prepared at the University of Canterbury and subsequently sent for extraction and purification at the Laboratory of Growth regulators and Department of Chemical Biology and Genetics. Extraction was performed on 3 to 5.5 mg DW of prepared biological triplicates in 1 ml of modified Bieleski solution (60% MeOH, 10% HCOOH and 30% H_2O). Purification was performed as described by Dobrev and Kamínek (2002) with minor modifications (Antoniadi et al. 2015). The samples were purified using a combination of C_{18} (100 mg ml^{-1}) and MCX cartridges (30 mg ml^{-1}), and purification recovery was validated by using 18 stable isotope-labeled cytokinin internal standards (0.2 pmol of cytokinin bases, ribosides, N-glucosides, 0.5 pmol of O-glucosides and nucleotides). Sample analysis was performed using the LC-MS/MS system consisting of an ACQUITY UPLC® System (Waters) and Xevo® TQ-S (Waters) triple quadrupole mass spectrometer. A multiple reaction-monitoring (MRM) mode of selected precursor ions and the appropriate production were used for quantification (Svačinová et al. 2012). Full cytokinin profiling can be found in Appendix Table 3.1.

3.2.5 Statistical analysis

One-way ANOVA with Tukey correction was used to test the significance of differences in dry weight of regrown leaves at 168 h after cutting between growth conditions. The following variables were subject to a two-way ANOVA with Tukey correction to test the effect of the growth condition at time 0 including N content, C content, C: N ratio and total WSC. Sidak correction was used for the individual amino acids to test relative differences in concentration between time 0 and 8 h after defoliation, and between uncut and cut plants at 24 h and at 168 h after defoliation. Cytokinin content was analysed by T-test with two-tailed distribution and homoscedasticity to compare cut plants at 8 h after defoliation and control uncut plants.

3. 3 Results

3.3.1 Physiological responses to contrasting nitrogen supply and carbohydrate loads

To evaluate the effect of contrasting internal carbohydrate status and contrasting NO_3^- supply on perennial ryegrass plants, physiological measurements, total internal C and N content, total WSC abundance, and total amino acid concentration were determined at the start of the experiment (t = 0 h). Perennial ryegrass plants were initially grown under N-sufficient conditions, subsequently exposed to contrasting day length (continuous light, D or 8 h light: 16 h dark, d) and contrasting NO_3^- supply (5 mM, N or 50 µM, n) for two weeks, and then harvested over time following defoliation. Production of foliage, tillers and regrowth leaf material at 168 h after defoliation was more strongly related to C rather than to N supply (Fig. 3.1A, B, C). Indeed, fewer green leaves (Fig. 3.1A), fewer tillers (Fig. 3.1B), and a lower DW of regrown leaf (Fig. 3.1C) were found under d relative to D, regardless of the N supply (i.e. dN or dn).

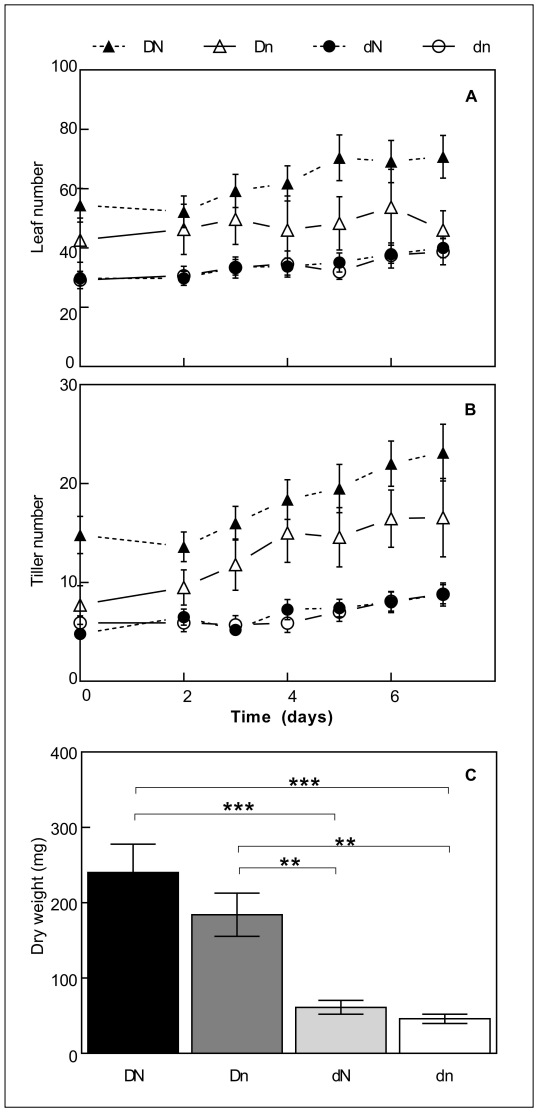


Figure 3.1. Variation over time in green leaf number (A) and tiller number (B) of the uncut perennial ryegrass plants grown under either continuous light (referred as D) or short days (8 h light: 16 h dark, d), and watered with N-free Hoagland medium modified with either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source. Dry weight (C) was measured 168 h after defoliation on regrown leaf material (foliar/lamina material located above the sheath). Data are means ± SE, n=7 to 10. Asterisks in (C) represent a significant difference between growth conditions calculated by one-way ANOVA with Tukey correction (*, ** and *** correspond to P-values of $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively). Note that the difference in DW between DN and Dn, as well as between dN and dn is not significant.

The response of total N content across the four growth conditions correlated with the response of total amino acid concentration in the leaf and sheath tissues before defoliation (Fig. 3.2A, D).

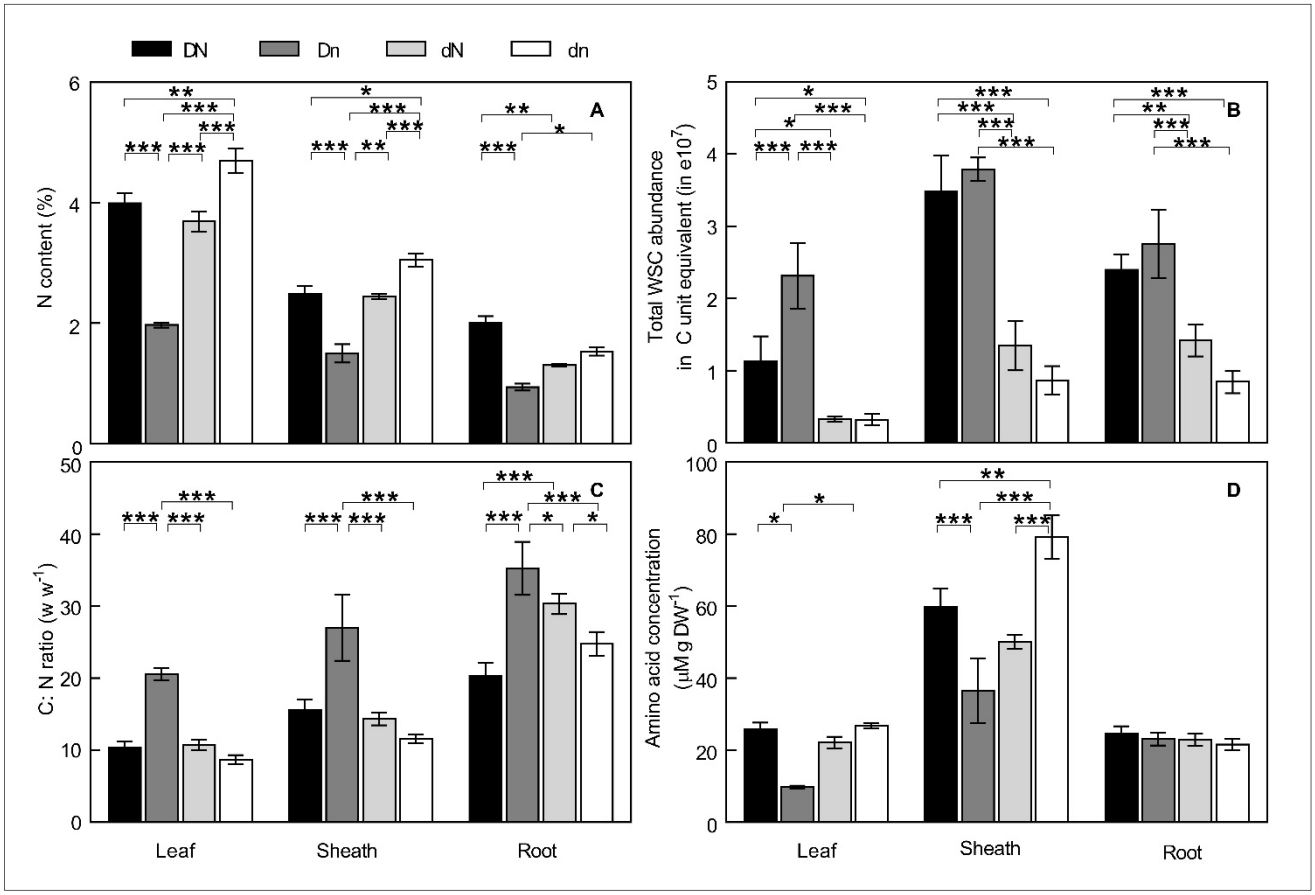


Figure 3.2. Total N content (A), relative abundance in C units of water soluble carbohydrate (WSC) content (B), C: N ratio (C) and total concentration in amino acids (D) in perennial ryegrass leaf, sheath, and root at time 0 (before defoliation). Plants were exposed to continuous light (referred as D) or short days (8 h light: 16 h dark, d), and watered with modified N-free Hoagland medium containing either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source. Data are means ± SE, n = 3 pools of seven plants. Asterisks represent a significant difference between growth conditions calculated by two-way ANOVA with Tukey correction (*, ** and *** correspond to P-values of $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively).

By contrast, total amino acid concentration in the root (Fig. 3.2D) did not vary across growth conditions. Total C content was not significantly different across growth conditions in the leaf and in the sheath, whereas significant changes were observed in the root between DN and

Dn, and between Dn and dN (Appendix 3.1). Exposure to D was associated with greater total WSC abundance (in C equivalents) than under d (Fig. 3.2B). In addition, Dn resulted in significantly greater WSC abundance in the leaves relative to DN (Fig. 3.2B). The C: N ratio was the greatest under Dn than it was under the three other growth conditions (Fig. 3.2C). Internal N content was relatively greater in the leaf, intermediate in the sheath and lowest in the root (Fig. 3.2A), whereas total WSC abundance and total amino acid concentration were greater in the sheath (Fig. 3.2B, D).

3.3.2 Defoliation–induced remobilisation of water soluble carbohydrates

To investigate the dynamics of remobilisation of the internal carbohydrate availability in response to defoliation, WSCs were recorded over time at 8 h, 24 h, and 168 h after cutting under growth conditions of contrasting carbohydrate status and NO_3^- supply.

The di- saccharides and the low-molecular weight (LMW) WSCs forms of tri-, tetra-, and penta-saccharides were the most abundant WSCs in terms of C unit equivalents (Fig. 3.3). Defoliation induced a general remobilisation of WSCs, which was initiated in the leaf as early as 8 h after cutting (Fig. 3.3B). The remobilisation of root WSCs was preceded by a slight increase in WSCs at 8 h (Fig. 3.3C). By 168 h after defoliation, the WSC profile differed across experimental conditions and across tissue types. In the leaf, most of the LMW WSCs, here defined as oligosaccharides of degree of polymerisation (DP) from DP3 to DP9, had recovered to their pre-cut values 168 h after defoliation, regardless of growth conditions (Fig. 3.3A). At 168 h after cutting, the sheath LMW WSC levels had also recovered pre-cut levels under D. However, under the Dn treatment, the sheath LMW WSCs had accumulated to greater levels than those recorded at 0 h (Fig. 3.3B). By contrast to D, exposure to d was associated with a progressive remobilisation over time of the sheath WSCs, which resulted in the total depletion of most of the sheath HMW WSCs (DP10-20) under d by 168 h (Fig. 3.3B). Similarly, most of the root WSCs reserves were exhausted by 168 h after defoliation (Fig. 3.3C). The remobilisation of the WSCs over time was relatively faster under conditions of imbalanced C/N ratio, such as Dn and dN conditions: e.g., for the LMW WSCs under Dn and dN conditions, and for the HMW WSCs under dN (Fig. 3.3B).

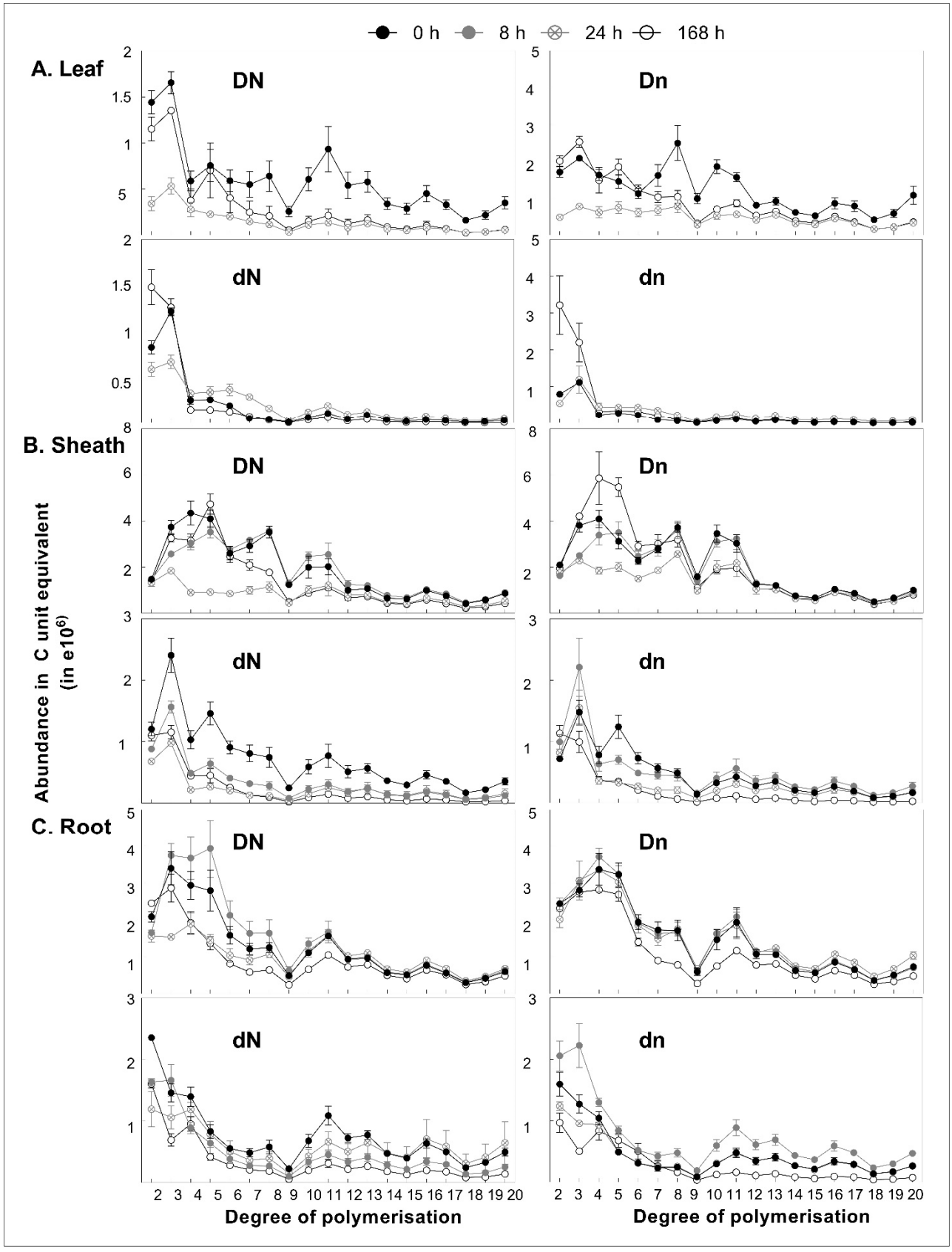

0 h
8 h
24 h
168 h
A. Leaf
B. Sheath
C. Root
DN
Dn
dN
dn
Abundance in C unit equivalent (in e10(6))
Degree of polymerisation
Degree of polymerisation

Figure 3.3. Remobilisation of WSCs over time after defoliation in the leaf (A), sheath (B) and root (C) of perennial ryegrass plants exposed to continuous light (D) or short days (8 h light: 16 h dark, d), and watered with modified N-free Hoagland medium containing either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source. Determinations were made at 0 hours (time of defoliation), and 8 h, 24 h, and 168 h after defoliation. Leaf material at 8 h after defoliation had not regrown yet and could not be harvested. Data are means ± SE, n = 3 pools of seven plants.

3.3.3 Amino acid profiles

Nitrate assimilation into organic compounds was estimated by measurement of the abundance of individual free amino acids (Fig. 3.4).

Some of the amino acids varied significantly in response to defoliation and some also changed over time in the control uncut plants, although generally to a lesser extent than in response to defoliation (Appendix 3.2). Overall, a significant increase was observed 24 h after defoliation and a subsequent decrease was noted at 168 h (relative to the same time point in control uncut plants). At 8 h after defoliation, under dn conditions the concentrations of Glu, Ile, Leu, Phe, Tyr, and Val were significantly greater in the roots of cut plants than in uncut plants at time 0. At 24 h, and 168 h after defoliation, the defoliation-induced changes in amino acid concentrations became significant, regardless of the tissue type and growth condition. In particular, at 24 h after cutting, Asp, Gly, His + Gln, Ile, Leu, Lys, Phe, Ser + Asn, Thr, Tyr, and Val increased significantly, whereas Glu, and Pro decreased compared to the uncut plants. The extent of the response to defoliation differed between growth conditions: it was greater under dN and dn conditions, and notably reduced under Dn. By 168 h after cutting, most of the changes observed under DN conditions took place in the root, whereas a significant decrease in amino acids was recorded mainly in the sheath and leaf under dN and dn.

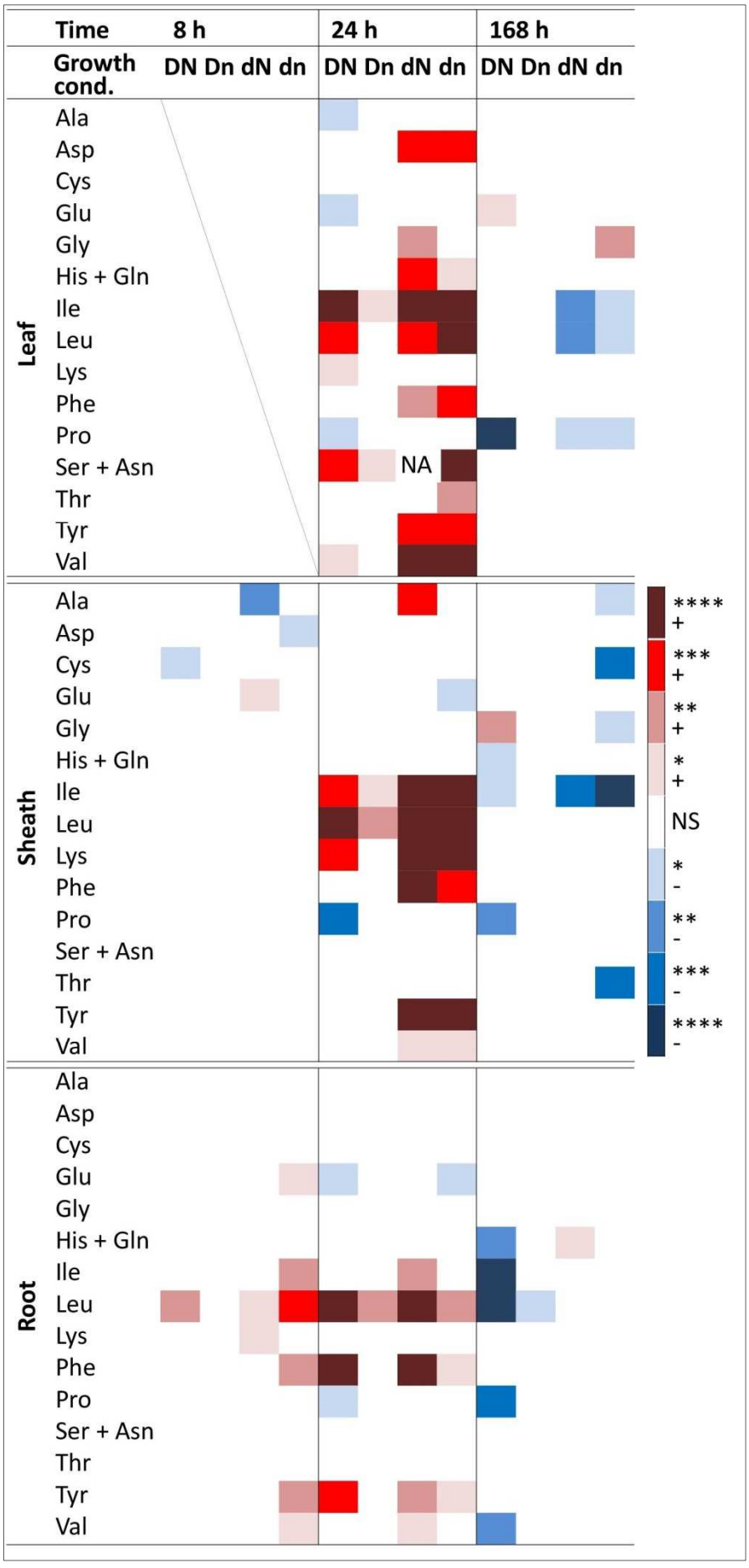

Time
8 h
24 h
168 h
Growth cond.
DN Dn dN dn
DN Dn dN dn
DN Dn dN dn
Leaf
Sheath
Root
Ala
Asp
Cys
Glu
Gly
His + Gln
Ile
Leu
Lys
Phe
Pro
Ser + Asn
Thr
Tyr
Val
NA
**** +
*** +
** +
* +
NS
* -
** -
*** -
**** -

Figure 3.4. Heat map showing positive (red) or negative (blue) changes relative to uncut plants in response to defoliation for individual amino acid concentrations in the leaf, sheath and root of perennial ryegrass plants at 8 h, 24 h, and 168 h following defoliation. Plants were exposed to continuous light (D) or short days (8 h light: 16 h dark, d), and watered with modified N-free Hoagland medium containing either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source. Data are means ± SE, n = 3 pools of seven plants. Asterisks at 8 h indicate a significant difference between plant materials harvested 8 h after cutting and amino acids concentrations measured at 0 h (pre-defoliation). Significance in individual amino acid concentration at 24 h and 168 h in response to defoliation were relative to measurements at 24 h and 168 h in control (uncut) plants. The color gradient corresponds to the level of statistical significance calculated by two-way ANOVA with Sidak correction for the individual amino acids (*, **, *** and **** correspond to P-values of $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, respectively).

3.3.4 Cytokinin content eight hours after cutting

Most of the significant changes in cytokinin content observed at 8 h after cutting took place in the sheath rather than in the root (Fig. 3.5).

Defoliation was associated with an increase in the content of iP and iPRMP in the sheath, by more than 60 and 40% respectively, regardless of growth conditions (Fig. 3.5A). In contrast, a significant decrease in *t*ZRMP levels was recorded in sheath and root tissues under dn, and in the sheath of plants exposed to DN. More complex trends in the *t*ZR profiling were recorded: a decrease in the sheath under dN, and an increase in root *t*ZR under Dn, DN, and dN following defoliation. Most of the *cis*-cytokinin responses to defoliation were detected under dN. In particular, under dN, *c*ZRMP levels accumulated in the sheath and the root, *c*Z levels decreased in the root, and *c*ZROG accumulated in the root. Under dn a significant increase in *c*ZR was observed in the sheath following defoliation.

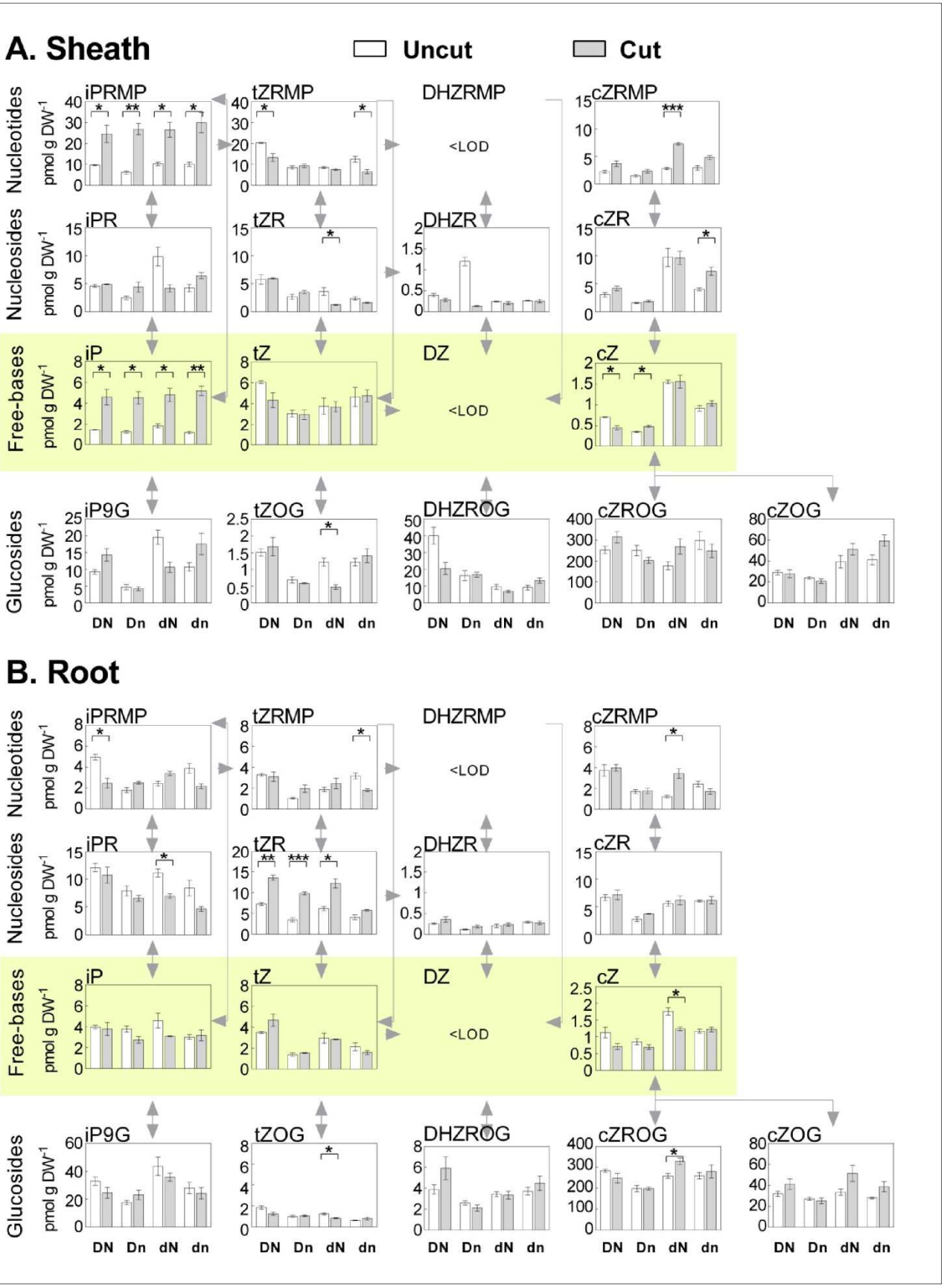

A. Sheath
Uncut
Cut
Nucleotides
Nucleosides
Free-bases
Glucosides
pmol g DW-1
iPRMP
tZRMP
DHZRMP
cZRMP
iPR
tZR
DHZR
cZR
iP
tZ
DZ
cZ
iP9G
tZOG
DHZROG
cZROG
cZOG
<LOD
DN
Dn
dN
dn
B. Root

Figure 3.5. Cytokinin content of perennial ryegrass sheath (A) and root (B) in control uncut plants (white bars) or in cut plants at 8 h after defoliation (grey bars). Plants were exposed to continuous light (D) or short days (8 h light: 16 h dark, d), and watered with modified N-free Hoagland medium containing either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source. Data are means ± SE, n = 3 pools of seven plants. Asterisks indicate a significant difference between cytokinin concentrations at 8 h after cutting and at 0 h (pre-defoliation). Statistical significance was calculated using a T-test (*, ** and *** correspond to P-values of $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively). Grey arrows represent conversions between cytokinin forms. The complete dataset can be found in Appendix Table 3.1.

3. 4 Discussion

3.4.1 Balance between carbon and nitrogen regulates nitrate assimilation of the leaf, sheath, and root of uncut plants

Identifying the partitioning of C and N reserves in perennial ryegrass is a key factor towards understanding the regulation of N assimilation. Distinct physiological characteristics reflect functional differences between tissue types. Namely, the sheath is the main site of WSC accumulation (Prud'homme et al. 1992) and amino acid synthesis and/or accumulation (Bigot et al. 1991), whereas N accumulates to a greater overall level in the leaf relative to the other tissues (Moser et al. 1982). Leaf N accumulates from either: (a) N forms directly taken up by the root, transported via xylem and stored in the leaf vacuoles (Granstedt and Huffaker 1982); or (b) complex N-containing molecules derived from the amino acids locally produced in the leaf or imported from the large pool of amino acids present in the sheath (Fig. 3.2A, D).

Previously, Morvan-Bertrand et al. (1999) showed that four days of exposure to contrasting photoperiodic conditions (continuous light or darkness, 16 h day: 8 h night cycle,) as an experimental treatment resulted in differential accumulation of carbohydrate stores and rapidly affected NUE of perennial ryegrass plants (Morvan-Bertrand et al. 1999). Growth under the experimental conditions described in section 3.2.1 resulted in contrasting internal N and C accumulation at time 0 (Figs. 3.2A, B, C and 3.3). Water-soluble carbohydrate accumulation, and in particular DP3-6, was dependant on the extent of the photosynthesis but was not significantly affected by N supply in the sheath or root (Figs. 3.2B and 3.3). By contrast, endogenous N content, and amino acid content varied with the interaction between

N supply and C demand within each tissue type, although the amino acid content in roots was not responsive to growth conditions (Fig. 3.2A, D). This indicates a constitutive accumulation and/or assimilation of amino acids in the root which occurs irrespective of the availability in N and C.

Comparing DN plants with Dn plants revealed that a greater N availability to the root correlated with a rapid consumption of photosynthetic C and with a lower WSC accumulation in the leaf, which could result in a relatively greater amino acid synthesis in the leaf and in the sheath tissues (Figs. 3.1A and 3.2B, D). This suggests a systemic integration of the C and N signals of supply and demand to support efficient amino acid synthesis. Consistently, plants grown under Dn conditions were exposed to a greater imbalance between C and N availability relative to the other treatments, which resulted in greater WSC accumulation and reduced accumulation of amino acids relative to DN, dN and dn (Fig. 3.2B, C). Lattanzi et al. (2005) found that long-term WSC stores had little impact on leaf growth, which suggests that the pool size of the WSC recorded under the experimental conditions of this experiment might result from N regulation of WSC accumulation. The data presented in Figure 3.3A support Lattanzi et al. (2005) observations that leaf growth relies mainly on the short-term supply of LMW WSCs and recent photosynthetic C in these uncut plants (Lattanzi et al. 2005). Therefore, the dynamics of WSCs remobilisation are coordinated with N partitioning to support N assimilation and subsequent growth response in perennial ryegrass.

3.4.2 Remobilisation of low- and high-molecular weight WSCs in the leaf and sheath support amino acid synthesis for rapid regrowth following defoliation

In the work, the spatiotemporal profiling of WSCs and amino acids in defoliated plants indicates three distinct patterns at 8 h, 24 h, and 168 h following an increase in defoliation-induced C demand from stored pools. At 8 h after defoliation, roots in plants exposed to dn presented an accumulation of WSCs across all DPs relative to time 0 (Fig. 3.3C), whereas the LMW WSCs were consumed in the sheath (Fig. 3.3B). Therefore, under low C and low N conditions, defoliation induced a remobilisation of the WSC reserve in the above-ground part of the plant and export to the roots for rapid assimilation into amino acids at the site of N uptake (Figs. 3.3B, C and 3.4). It is possible that the significant response observed 8 h after defoliation under conditions of limited external N availability and high C demand corresponds to a critical survival response to herbivory/defoliation characterised by greater

rate of N assimilation and/or remobilisation of initial N reserves relative to the other growth conditions (Figs. 3.2A and 3.4).

Ourry et al. (1990) showed that N taken up after defoliation was not directly assimilated but stored mainly in the vacuole of the leaf, sheath, and root tissues for up to five days after defoliation. This was followed in time by a period of N reduction throughout the plant between five and 12 days after defoliation (Ourry et al. 1990). This contrasts with the findings presented in Figure 3.4, which indicate that by 24 h following defoliation a marked accumulation and storage of amino acids had occurred. The discrepancy in the timing of the response to defoliation could be explained by greater initial C reserves under the experimental conditions presented in section 3.2.1 stimulating an earlier assimilation into amino acids relative to the plants in the Ourry et al. (1990) study. In addition, the data presented in Figure 3.4 indicate that the regrown leaf tissue and the sheath became the main sites for significant accumulation of amino acids in response to defoliation relative to uncut plants. Notably, by 24 h the LMW WSCs and most of the HMW WSCs were remobilised in the leaf and sheath to their lowest levels relative to the other time points (Fig. 3.3A, B). Together with WSC remobilisation, C can be used directly from photosynthesis of newly regrown tissues, with a proportional increase in photosynthate use compared with C reserves over time following defoliation as more photosynthetic tissues develop (Morvan-Bertrand et al. 1999).

The amino acids Glu, Gln, Asp, Asn are direct products from the GS-GOGAT pathway and are the precursors of the other amino acids. The changes in Gln, Asp, Asn in response to defoliation were more significant in the leaf tissue than in the sheath and the root 24 h after defoliation (Fig. 3.4). This suggests that removal of leaf material might have shifted the main site of amino acid assimilation and/or accumulation from the sheath at time 0 to the growing leaf 24 h after defoliation (Figs. 3.2D and 3.4). In addition, the significant response to defoliation in the leaf was extended to most of the individual forms of amino acids, whereas most of the changes in the sheath were observed for Ile, Leu, Lys, Phe and Tyr relative to uncut plants at 24 h (Fig. 3.4). This suggests that defoliation not only stimulated N assimilation in the leaf but also the subsequent conversion into complex N-containing molecules to support regrowth of photosynthetic leaf material in the 24 h period after defoliation.

Rapid regrowth after defoliation relies on complementary fluxes of newly-acquired and reserve-derived C and N, and is associated with re-establishment of a balanced C/N ratio from complementary fluxes of reserve-derived and currently assimilated C and N (Richards and Caldwell 1985, Ourry et al. 1989, Schnyder 1999). Greater initial WSC reserves recorded under Dn at time 0 (Fig. 3.2) was associated with relatively faster remobilisation and recovery of LMW WSC when compared with the other growth conditions in response to defoliation (Fig. 3.3) due to a limitation in N availability for C assimilation (Fig. 3.4).

Louahlia et al. (2008) suggested that the concentration of amino acids in defoliated perennial ryegrass increased for up to 24 h and then decreased (Louahlia et al. 2008). Indeed, at 168 h after defoliation, the data presented in Figure 3.3A suggests that a negative feedback reduced the defoliation-induced accumulation of free amino acids observed 24 h after cutting. Nitrogen reduction during regrowth may originate from either root N uptake or from remobilisation through proteolysis and/or amino acid hydrolysis in a species dependant proportion (Thornton et al. 1993). In addition, Prud'homme et al. (1992) identified two-phases in WSC dynamics following defoliation: the WSCs are remobilised in all tissues for up to six days following defoliation to sustain foliage development, after which a second period is characterised by a recovery of the WSCs stores levels for up to 29 days after defoliation (Prud'homme et al. 1992).

In this study, two highly significant decreases in amino acids were recorded at 168 h after defoliation relative to uncut plants: under dn in the sheath and under DN in the root (Fig. 3.4). By 168 h after cutting, the root WSCs from DP2-3 had partially recovered and DP6-20 WSCs had reached their lowest levels relative to the other time points under DN (Fig. 3.3C), whereas the sheath WSCs from DP7-20 were depleted under dn (Fig. 3B). The response to defoliation in DN roots corresponds to an active remobilisation of resources which could be associated with root growth. A limited availability of N and C under dn condition resulted in a relative longer remobilisation of the amino acids from the large N pool stored in the sheath at time 0 (Fig. 3.2D) when compared with DN, Dn and dN at 168 h after defoliation. By contrast, the leaf and sheath of plants grown under DN presented stabilised levels of amino acids (Fig. 3.4), and partially recovered WSC levels (Fig. 3.3A, B) as well as a greater growth response after defoliation relative to the other experimental conditions (Fig. 3.1C). This indicates that by 168 h after cutting the leaf and sheath were almost fully recovered from the C perturbation created by the defoliation treatment. The results of the research presented in

this chapter support the observations of Schnyder (1999) that foliage production rate after defoliation is related to C rather than N supply (Schnyder 1999).

3.4.3 Cytokinins respond to defoliation

Maintenance of a C/N balance requires local and long-distance signals to coordinate C and N supply and use across tissue types. The iP-type nucleoside form of cytokinin is considered to be a shoot to root signal of the N status of the plant, whereas the *t*ZRs could be a root to shoot signal of N availability (Hirose et al. 2008, Ruffel et al. 2011, Kiba et al. 2013). Defoliation under the experimental conditions described in section 3.2.1 resulted in significant accumulation of iP and iPRMP in the sheath across all growth conditions and *t*ZR accumulation in the root under DN, Dn and dN in response to defoliation in plants 8 h after cut (Fig. 3.5). This suggests long distance sheath to root and/or root to sheath signalling mediated by cytokinins taking place 8 h after defoliation (Fig. 3.5A). Considering that the dN condition is characterised by a limited C availability and high N supply, it is possible that cytokinins are induced by a decrease in the C/N ratio and act as early systemic signals for rapid regulation of WSC remobilisation and amino acid synthesis under such growth conditions. Gajdošová et al. (2011) hypothesised that *c*Z- containing tRNA maintains minimal levels of cytokinins under growth-limiting conditions (Gajdošová et al. 2011) and this could explain the significant changes in the content of *c*Z-type cytokinins measured under the experimental conditions of this experiment at 8 h after defoliation (Fig. 3.5).

3. 5 Conclusion

This chapter highlights the integration of the N and C signals for amino acid assimilation and regrowth following defoliation. Therefore, the results discussed here extend the analysis presented in Chapter 2 by including a defoliation-induced C demand to perennial ryegrass plants. Taken together, the results presented in Chapter 3 led to the hypothesis that cytokinins act as a mobile signal between the leaf sheath and the root system regulated by the C/N ratio 8 h after defoliation. Subsequently, an overall increase in amino acid synthesis took place, mainly in the leaf and sheath, to support rapid regrowth of photosynthetic material 24 h after cutting. Finally, a negative feedback brought the response back towards pre-defoliation levels within 168 h and this resulted in contrasting amino acid profiling depending upon the initial

C and N reserves and availability during regrowth. This study indicates that there are substantial differences between tissue types and this develops an understanding of the spatial and temporal dynamics in C and N assimilation taking place following the removal of above-ground tissue likely to be associated with grazing. A better understanding of the underlying mechanisms controlling the efficiency of N uptake and assimilation is required if we are to limit N leaching in managed ryegrass pasture systems. In addition,

From the data acquired in Chapter 2 and 3, further investigation was now going to look at the potential use of exogenous cytokinin application to perturb N use efficiency in perennial ryegrass plants (see Chapter 4). Most commercial field applications of cytokinin-like compounds as a biotechnological tool enhancing productivity have targeted fruit tree, vegetable, and cereal crops (Koprna et al. 2016). Chapter 4 presents dose-response experiments investigating the potential positive effects of exogenous treatments of CPPU, a cytokinin-like compound, on perennial ryegrass NUE. In addition, Chapter 4 aimed at developing a molecular understanding of the relative implication of cytokinin signalling pathway in response to hydroponic treatment with high concentrations of N and PI-55, only compound reported to act as a competitive antagonist of cytokine receptors.

Chapter 4

Evaluating the effectiveness of CPPU and PI-55 in moderating the nitrogen use efficiency of *Lolium perenne*

Contents

4. 1 Introduction

Drawing on the findings of Chapters 2 and 3, endogenous cytokinins were shown to be involved in the response of perennial ryegrass (*lolium perenne L.*) to nitrate (NO_3^-) addition and were hypothesised to act as a long-distance signal regulated by the carbon (C) to nitrogen (N) ratio of the plant as early as 8 h after defoliation, preceding the remobilisation of the water-soluble carbohydrate (WSC) stores.

The naturally occurring cytokinins are plant growth regulators with the ability to act in a dose-dependent manner to control many aspects of plant growth (see Section 1.4.1).
A developing understanding of the activity of cytokinins in non-model plant species, together with a better understanding of the cytokinin signalling pathway, has been associated with commercial interest in manipulating endogenous cytokinin metabolism, transport, and perception as a biotechnological tool to improve agricultural traits and help reduce N fertiliser input levels to the field (Isogai et al. 1981, Capelle et al. 1983, Wang et al. 2011). Generally, when exogenous applications of cytokinins have been used to improve yield traits, either kinetin or 6-benzylaminopurine (6-BAP) have been used (Koprna et al. 2016) (see Fig. 4.1 for structure).

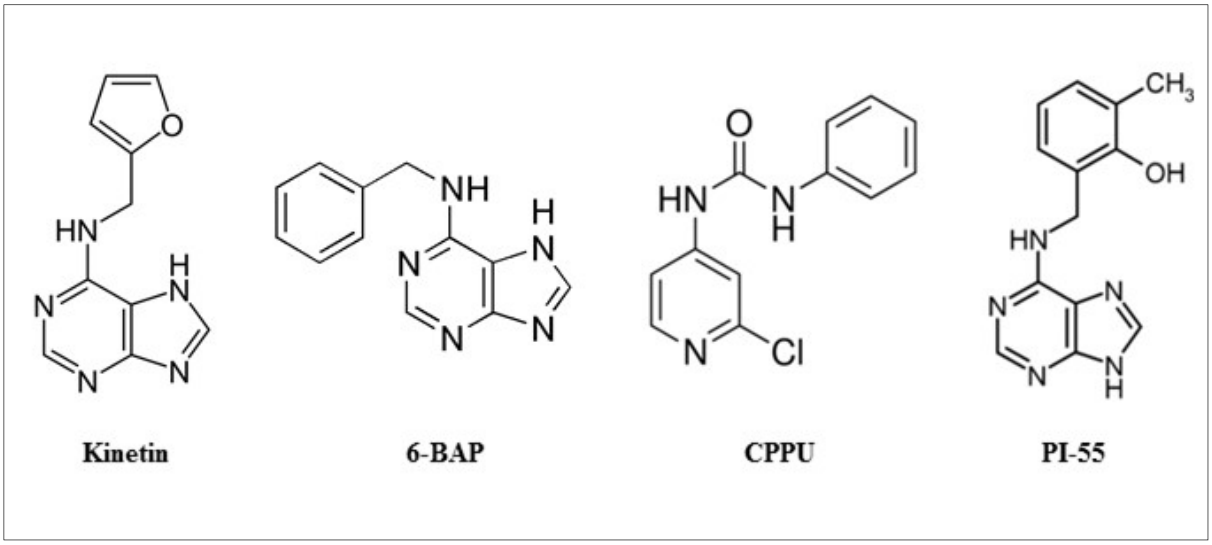


Figure 4.1. Structure of compounds with cytokinin activity: kinetin, 6-benzylaminopurine (6-BAP), *N*-(2-chloro-4-pyridyl)-*N*′phenylurea (CPPU), and cytokinin receptor antagonist 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55).

In addition, *N*-(2-chloro-4-pyridyl)-*N*′phenylurea, subsequently referred to as CPPU, has been patented as a cytokinin-like compound with the most potent plant growth regulating properties relative to the other compounds shown in Figure 4.1 (Isogai et al. 1981). However, most commercial field applications of CPPU have targeted fruit, including kiwifruit (Patterson et al. 1993), watermelon (Hayata et al. 1995), grape (Nickell 1985), and apple (Bangerth and Schröder 1994), and their potential for improving N use efficiency (NUE) has yet to be studied in perennial ryegrass.

Another approach to manipulate the endogenous cytokinin content is by treatment with chemical inhibitors of cytokinin action. To date, only a few compounds antagonistic to cytokinin receptors have been identified, among which is 6-(2-hydroxy-3-methylbenzylamino)purine, designated PI-55 by Spíchal et al. (2009) (Fig. 4.1). This purine derivative competitively inhibited the binding of the natural ligand *trans*-zeatin (*t*Z) onto its receptors in transformed *Escherichia coli* strains expressing the *Arabidopsis thaliana* cytokinin receptors referred to as cytokinin response 1 (CRE1)/ *A. thaliana* histidine kinase (AHK) 4 and AHK3 (Spíchal et al. 2009). Applications of PI-55 to *A. thaliana* seedlings was able to perturb root growth and accelerated seed germination (Spíchal et al. 2009). Cytokinin bioassays revealed that PI-55 inhibited cytokinin-dependent biological responses such as cell division in *Nicotiana tabacum* callus, retention of chlorophyll in excised *Triticum aestivum* leaves, and betacyanin formation in *Amaranthus caudatus* seedlings at micromolar concentrations (Spíchal et al. 2009). This compound was also used in the present study.

The first objective of this study was to evaluate the effect of exogenous application of CPPU, as a biotechnological tool to improve perennial ryegrass regrowth following nitrate application and defoliation. For this purpose, CPPU was applied one day before NO_3^- treatment and defoliation of perennial ryegrass plants with the hypothesis that exogenous cytokinin treatment mediates a signal "priming" the remobilisation of water-soluble carbohydrate (WSCs) reserves required for NO_3^- assimilation. Plant growth measurements included shoot and root biomass at seven days after treatment with 5 mM KNO_3, tiller number, leaf number, leaf regrowth rate, maximum photosynthetic rate A_{max}, and stomatal conductance g_s over a 28 day period.

The second objective was to investigate the involvement of the cytokinin signalling pathway in the physiological response of the plant to NO_3^- addition by inhibiting the cytokinin

receptors. This was done by hydroponic treatment of perennial ryegrass plants with PI-55 and 6-BAP on d_{n-1} and subsequent treated with 5 mM KNO_3 on d_n. The activation of the cytokinin signalling pathway in response to 5 mM KNO_3 treatment was determined by monitoring the relative expression of cytokinin response regulators, Type-A and Type-B *LpRR* gene family members, following PI-55 treatment, with the hypothesis that PI-55 should inhibit the N-induced cytokinin signalling/relay system.

4. 2 Material and methods

4.2.1 Plant growth

In order to test the effect of exogenous cytokinin application and endogenous cytokinin perturbations on perennial ryegrass plants, glasshouse experiments were conducted at the University of Canterbury glasshouses (43^{O}31'48" S, 172^{O}37'13" E). Room temperature was maintained at 22^{O}C temperature by roof ventilation and soil water content was maintained by automatic watering for five minutes daily by mist emitters located throughout the room. *Lolium perenne* L. cv. "Grasslands Nui" seedlings were grown using unfertilised bark-free soil commercially acquired near Christchurch, New Zealand. Small trays (420 mm x 300 mm) were sown with 120 – 140 seeds in four rows of 30 – 35 seeds each, and plants maintained for five months until treatments by foliar application of CPPU. CPPU was dissolved into 95% ethanol and then dispersed into water to obtain a stock solution of 40 µM CPPU. Known amount of stock solution were added into water to obtain the CPPU solutions at the final concentration of 0.4, 4, 20 or 40 µM CPPU. At time of application, 0.01% Tween® 20 was added to the CPPU solutions. Control plants were sprayed with a solution containing 0.01% Tween® 20 and water instead of CPPU.

Time zero of the experiment was defined by immersion of the trays into a solution of 5 mM of KNO_3 for one minute and immediate defoliation at 4 cm above ground level which took place one day after CPPU treatment. A subset of the trays were used for cycles of weekly CPPU treatments on d_{n-1} and subsequent N treatment/ cut on d_n. The initial set of treatments was referred to as cycle 1.

Another set of perennial ryegrass seeds were sown on individual pots and used for the PI-55 experiment. Single perennial ryegrass seeds were germinated in Eppendorf tubes (tube tips

cut) filled with perlite and placed in soil until plant establishment. Eleven-week old seedling roots were washed from soil and their tubes transferred to polyvinyl chloride channels in the homemade hydroponic system. Seedlings were maintained for one week in N-free Hoagland liquid medium (BioWorld, USA) for acclimation to liquid growth conditions. Treatments with solutions of 6-BAP at 1 µM dissolved in 0.1% dimethyl sulfoxide (DMSO), or with solutions of PI-55 at 0, 0.01, 0.1, 1, or 10 µM concentrations dissolved in 0.1% DMSO were applied six days after transfer to hydroponics by direct addition into the liquid medium. Control plants were treated with water and 0.1% DMSO. On the following day (d0) perennial ryegrass plants received 5 mM of KNO_3 to the medium. Temperature, pH, and electrical conductivity of the medium were monitored daily and, when required, the water level and pH were adjusted. Measurements described in Sections 4.2.2, 4.2.3, and 4.2.4 were performed at the University of Canterbury (Christchurch, New Zealand).

4.2.2 Physiological measurements

Plant material was oven dried at 65°C for three days and the biomass determined. Repeated measures were made of tiller number, leaf number, and leaf elongation rate was determined. Leaf-level gas exchange measurements were made on fully expanded, newly mature leaves of two to three plants per treatment per time point using two cross-calibrated Licor 6400 photosynthesis system (Li-6400, Li-Cor Inc.) with CO_2 control and the standard 2 by 3 cm chambers, each equipped with a blue-red light source. Measurements were performed with the following settings: relative humidity of 60 – 70%, CO_2 reference of 400 ppm, chamber temperature at 20°C, and photosynthetic photon flux density (PPFD) of 1500 µmol m^{-2} s^{-1}. Maximum photosynthetic capacity A_{max} and stomatal conductance g_s were recorded when CO_2 and H_2O concentrations in the sample infrared gas analyser (IRGA) had stabilised (typically within 20 min of exposure to 1500 µmol m^{-2} s^{-1} PPFD) at light saturating rate of 400 ppm of CO_2 at 20°C.

4.2.3 RNA isolation and cDNA synthesis

Root and shoot samples from five plants were pooled upon harvest, immediately flash frozen, stored at -80^{O}C, and treated as one biological replicate. Up to 100 mg of frozen plant material were ground under liquid nitrogen into a thin powder and used for extraction of total RNA using RNeasy Plant Mini Kit (Qiagen, Germany), and RNase-Free DNase Set (Qiagen,

Germany) to prevent genomic DNA contamination. Total RNA integrity and quality were checked using a Nanodrop™ spectrophotometer and by electrophoresis on 1% (w v^{-1}) agarose gel. Reverse transcription of RNA to cDNA required approximately 1 µg of total RNA, 50 U Expand Reverse Transcriptase (Roche, Mannheim, Germany), 50 pmol oligo (dT) primers, and 100 pmol random hexamer (pdN6) primers in a 20 µL reverse transcription reaction. The reaction mix was kept at room temperature for 10 min, incubated at 42°C for 60 min, and subsequently placed at 70°C for 15 min to deactivate the enzyme.

4.2.4 Quantitative reverse transcription polymerase chain reaction

Specific PCR primers for each of the *LpRR* gene family members were the same as used in Chapter 2 and used to measure the relative expression level by quantitative RT-qPCR. All RT-qPCR reactions were performed in a Rotor-Gene Q system real-time PCR instrument (Qiagen Hilden, Germany), using a 20 µL reaction mix containing 1 µL of 10-fold diluted cDNA, specific forward and reverse primers, and home-made SYBR Green master mix (Song et al. 2012). The reference genes, elongation factor 1 alpha (*EF*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were used as internal controls to normalise the expression level of *LpRR* gene family members using the $2^{-\Delta\Delta Ct}$ method modified as described previously by Song et al. (2012). Three technical replicates were carried out for one sample set of five pooled plants. Root and shoot expression were analysed as two independent sample sets. Relative expression (fold changes) for each of the *LpRR* gene family members was calculated relative to the lowest expressed sample of the sample set for each treatment type (control, 6-BAP or PI-55), and was corrected using the geometric means of the reference genes *EF* and *GAPDH*. 0= pre-treatment, and 1 and 7 for one and seven days after N addition respectively. *Lp2138* and *Lp2183* correspond to two sets of primers designed for the same gene *LpRR12a*. Fold change from *Lp2138* and *Lp2183* relative expression were averaged to calculate *LpRR12*a expression (Tables 4.1, 4.2 and Fig. 4.8). The complete dataset can be found in Appendix Tables 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6.

4.2.5 Statistical analysis

The variables were subjected to a two-way ANOVA with treatment type (CPPU at 0, 0.4, 4, 20, 40 µM, or PI-55 at 0, 0.01, 0.1, 1, 10 µM, and6-BAP 1 µM) and time (0 = immediately before N treatment and cut, and 1, 3, 5, 7, 14, 21, 28 for one, three, five, seven, 14, 21, and 28

days after N addition and cut respectively, or cycle 1, 2, 3, 4 for one, two, three, and four regrowth cycles respectively) as the factors. Significant effects were defined by Tukey's multiple comparisons test. Results of an ANOVA were considered statistically significant when P-values ≤0.05. One-way ANOVA with Tukey's multiple comparisons was used to test the biomass of regrown leaf material after a single treatment with various concentrations of CPPU (Fig. 4.2).

4. 3 Results

4.3.1 Regrowth and photosynthetic responses to CPPU treatment

To understand the effect of CPPU on plant development and NUE, solutions of 0, 0.4, 4, 20, and 40 µM of CPPU were sprayed on perennial ryegrass one day before N treatment and defoliation and then measurements of biomass (Fig. 4.2), physiological (Fig. 4.3), and photosynthetic parameters (Fig. 4.4), were performed over time.

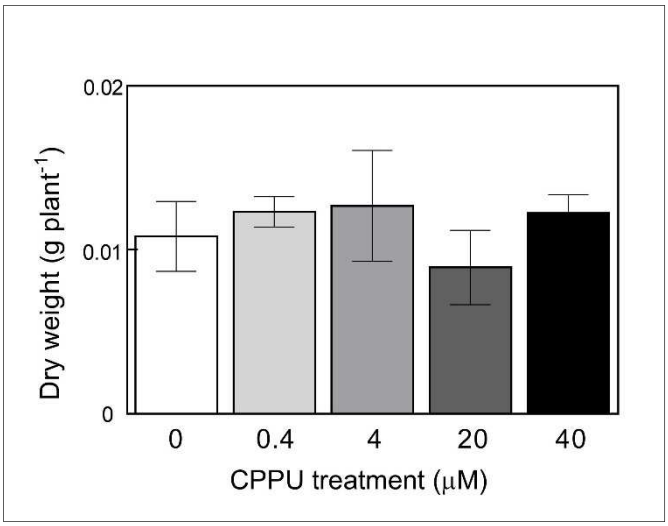


Figure 4.2. Effect of CPPU treatment on biomass of regrown leaf material seven days after defoliation. Plants were sprayed with increasing concentrations of CPPU, one day before treatment with 5 mM KNO_3 and defoliation at 4 cm above ground level. Control plants were sprayed with a solution containing water instead of CPPU. Data are means ± SE, n = 3 pools of 10 plants. Statistical significance was tested by one-way ANOVA with Tukey correction and resulted in P-values ≥0.05.

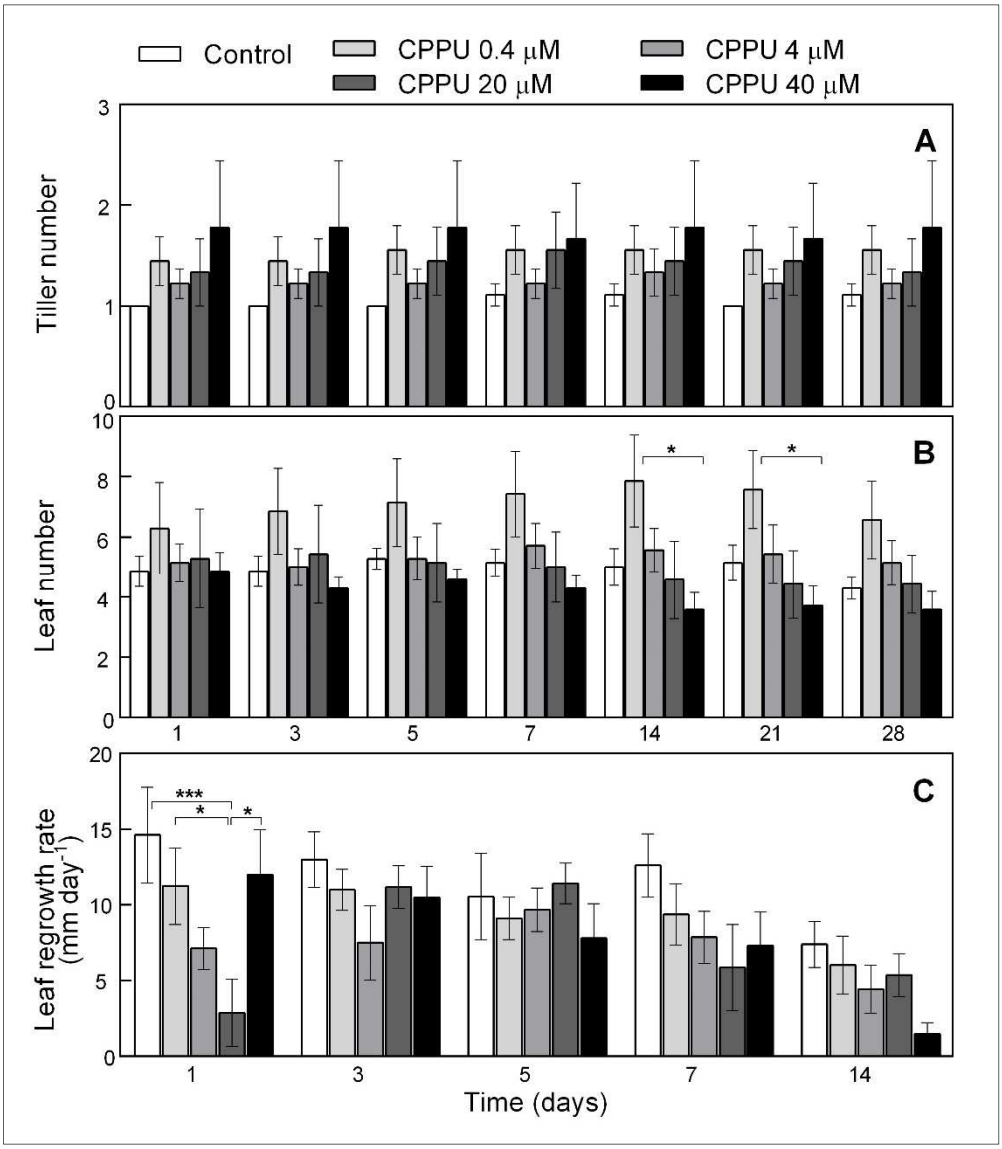


Figure 4.3. Physiological parameters of perennial ryegrass plants recorded in response to CPPU treatments. Tiller number (A), leaf number (B), and leaf regrowth rate (C) were measured over time following a single treatment with various concentrations of CPPU, one day before treatment with 5 mM KNO_3 and defoliation at 4 cm above ground level (d0). Control plants were sprayed with a solution containing water instead of CPPU. Data are means ± SE, n = 9 plants for tiller number; n= 7 for leaf number; n= 8 for leaf regrowth rate. Asterisks represent a significant difference between CPPU treatment for each time point determined by two-way ANOVA with Tukey correction (*, **, and *** correspond to P-values of $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively).

As a general trend, treatments with increasing CPPU concentration were associated with decreasing leaf number (Fig. 4.3B) and leaf elongation rate (Fig. 4.3C), although most changes were not statistically different to water. An early negative effect of CPPU treatment was associated with slower leaf regrowth of plants treated with 20 µM CPPU relative to the control plants and to the plant treated with 0.4 µM CPPU at d1 (Fig. 4.3C). Subsequently, a significant decreased in leaf number was noted between plants treated with 0.4 µM and 40 µM CPPU at d14 and d21 (Fig. 4.3B). Foliar application of CPPU did not significantly influence the tiller number of perennial ryegrass plants (Fig. 4.3A).

In order to investigate the putative effect of CPPU on photosynthesis, the maximum photosynthetic capacity A_{max} (Fig. 4.4A) and stomatal conductance g_s (Fig. 4.4B) were recorded over a 28 day regrowth period.

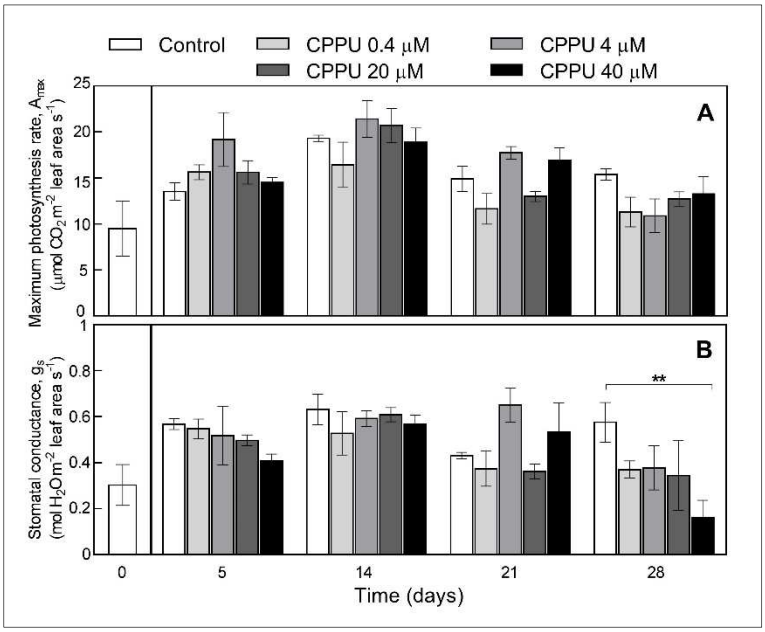


Figure 4.4. Maximum photosynthetic rate A_{max} (A) and stomatal conductance g_s (B) of plant treated with various concentrations of CPPU one day before treatment with 5 mM KNO_3 and defoliation 4 cm above ground level (d0). 5, 14, 21, 28 for five, 14, 21, and 28 days after N addition and cut respectively. Control plants were sprayed with a solution containing water instead of CPPU. Data are means ± SE, n = 3 plants; n= 2 for A_{max} at d21 after 0.4 µM CPPU treatment; n= 2 for g_s at d21 after control treatment. Asterisks indicate a significant difference between treatments for each time point calculated by two-way ANOVA with Tukey correction (*, **, and *** correspond to P-values of $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively).

The stomatal conductance g_s decreased at 28 days after treatment with 40 µM CPPU compared with control sprayed plants (Fig. 4.4B).

In order to manipulate the duration and extent of the physiological effects of CPPU after a single application (Figs. 4.2, 4.3 and 4.4), another experiment was carried with repeated cycles of weekly CPPU treatments on d_{n-1} and subsequent N treatment/ cut on d_n (Fig. 4.5).

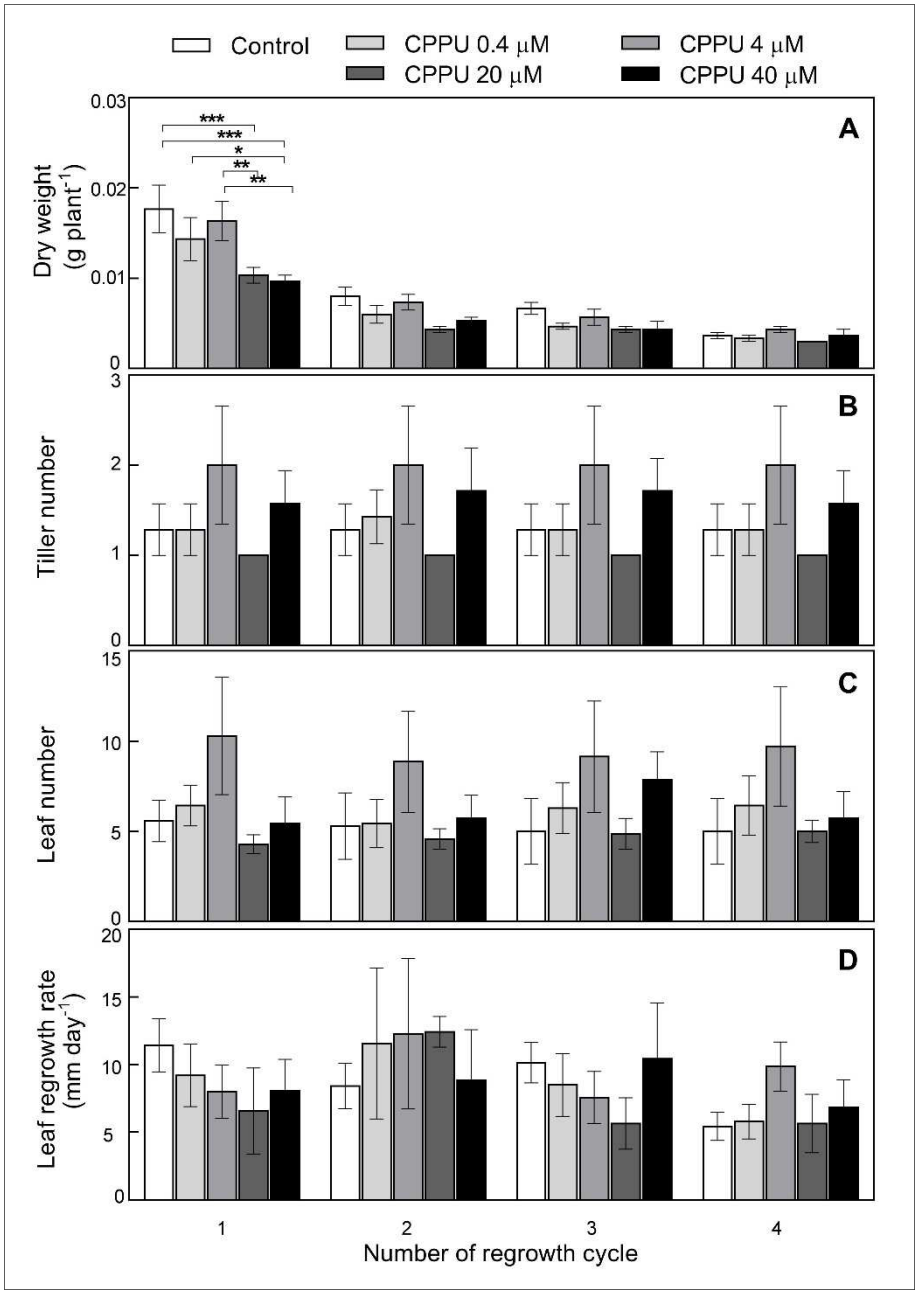


Figure 4.5. Dry weight (A), tiller number (B), leaf number (C), and leaf regrowth rate (D) were recorded over four cycles of N treatment and concomitant defoliation. Each cycle was defined by a pre-treatment at various CPPU concentrations one day before treatment with 5 mM KNO_3 and cut at 4 cm above ground level (d0). Treatment with CPPU was repeated at d6 and a new cycle was defined by the combined N-treatment, and cut on d7. Control plants were sprayed with a solution containing water instead of CPPU. Cycle 1, 2, and 3 corresponded to measurements performed on the seventh day of the cycle, immediately before a new N treatment and cut respectively. Cycle 4 corresponded to measurements recorded on the fifth day of the cycle. Data are means ± SE, n= 3 trays of 87 to 114 plants for dry weight; n= 7 for tiller number, leaf number, and leaf elongation rate. Asterisks indicate a significant difference between treatments for each time point and was calculated by two-way ANOVA with Tukey correction (*, **, and *** correspond to P-values of $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively).

Cyclic treatments were not associated with significant differences across CPPU concentrations in tiller number (Fig. 4.5B), leaf number (Fig. 4.5C) or leaf elongation (Fig. 4.5D). It is noteworthy that inconsistent measurements were recorded for the biomass of regrown plant material at d7: leaf dry weight was similar across treatments in a set of plants used for a single CPPU treatment (Fig. 4.2), whereas CPPU treatments at 20 and 40 µM were associated with a significant decrease in biomass relative to control measured with another set of plants used for the experiment of repeated treatments (Fig. 4.5A).

4.3.2 Effect of PI-55 treatments on growth response and expression of cytokinin response regulator genes

Hydroponic treatments of PI-55 and 6-BAP were performed to estimate the relative importance of cytokinins in the growth response of perennial ryegrass plants following N treatment. Figures 4.6 and 4.7 shows similar shoot and root dry weight at d7 (Fig. 4.6), tiller number (Fig. 4.7A), leaf number (Fig. 4.7B), and leaf elongation rate (Fig.4.7C) across treatment types at d0, d1, d3, and d7.

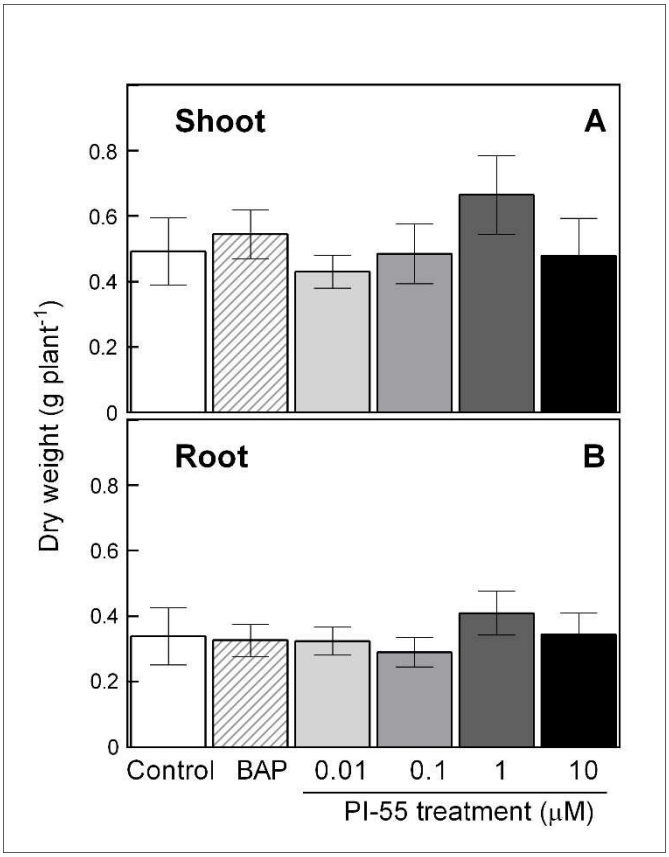


Figure 4.6. Dry weight of perennial ryegrass shoot (A) and root (B) at d7. Plants were hydroponically treated with 1 µM of 6-BAP or various concentrations of PI-55, one day before treatment with 5 mM KNO_3 (d0). Control plants were treated with water and 0.1% DMSO. Data are means ± SE, n= 9 to 10 plants. Statistical significance was tested between treatments for each tissue type by two-way ANOVA with Tukey correction and resulted in P-values ≥0.05.

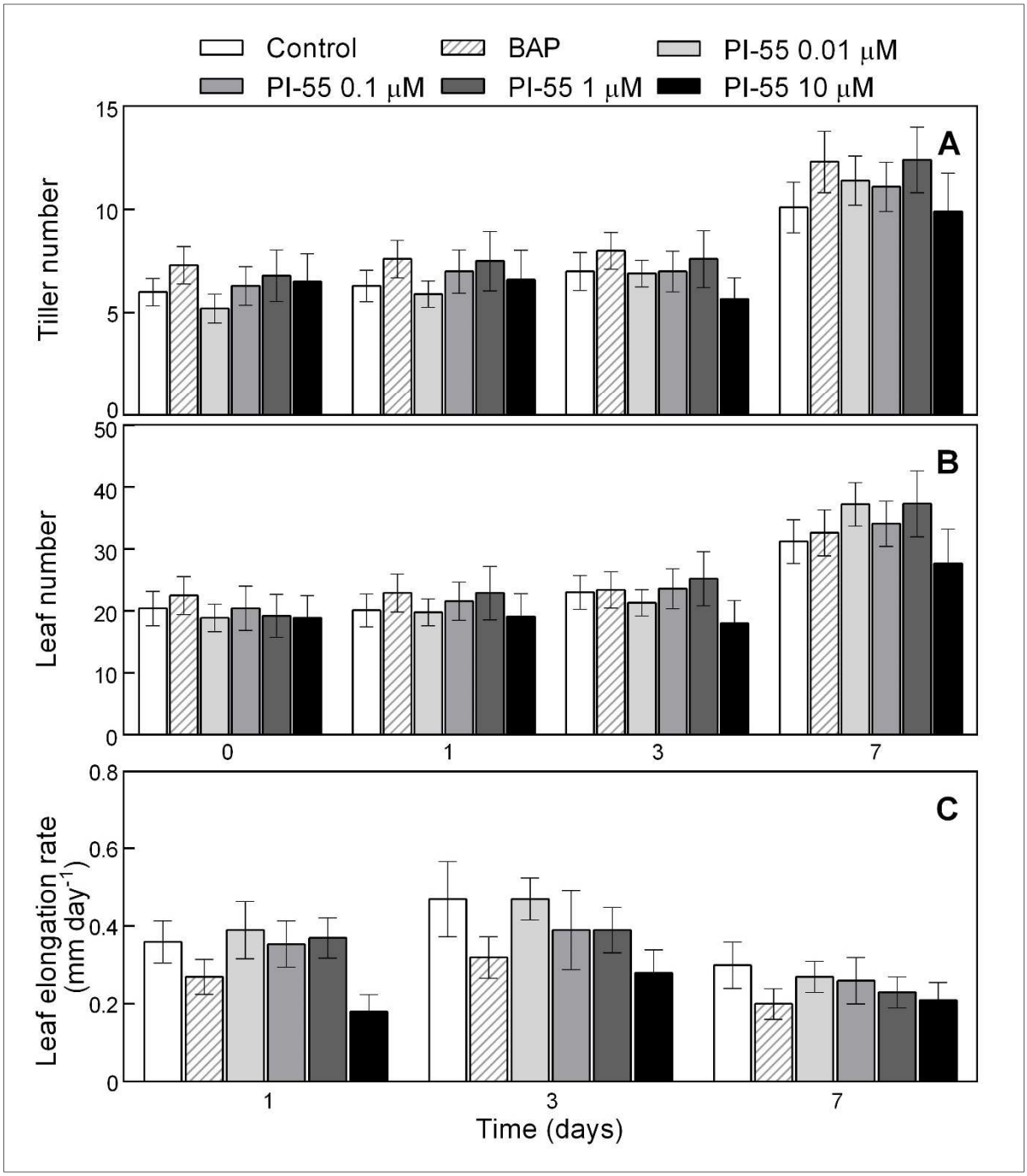


Figure 4.7. Variation over time in tiller number (A), leaf number (B), leaf elongation rate (C) of perennial ryegrass plants hydroponically treated with 1 μM of 6-BAP or various concentrations of PI-55, one day before treatment with 5 mM KNO_3 (d0). Control plants were treated with water and 0.1% DMSO. 0 =immediately before N treatment, and 1, 3, 7 for one, three, and seven days after N addition respectively. Data are means ± SE, n= 10 plants. Statistical significance was tested between treatments for each tissue type by two-way ANOVA with Tukey correction and resulted in P-values ≥0.05.

The relative gene expression of the *LpRR* gene family members was recorded in shoot and root tissue over time to determine whether the PI-55 treatment was able to antagonise the cytokinin signalling pathway. The fold change in expression was calculated for each *LpRR* gene family members in the shoots (Table 4.1) and in the roots (Table 4.2) of control plants treated with water and 0.1% DMSO.

Table 4.1. Relative expression of cytokinin response regulator (*RR*) gene family members in the shoot of perennial ryegrass after treatment with water and 0.1% DMSO (control) immediately before (d0) or at one (d1) or seven (d7) days after N treatment.

***RR* family members**		**Expression**	**d0**	**d1**	**d7**
Type-A *LpRRs*	*LpRR1*	Fold change	0.07	0.17	0.77
		SD	0.02	0.13	0.27
	LpRR3	Fold change	0.07	0.11	0.19
		SD	0.004	0.03	0.04
	LpRR6	Fold change	0.142	0.38	0.63
		SD	0.02	0.01	0.06
	LpRR9	Fold change	0.09	0.37	0.87
		SD	0.01	0.07	0.13
Type-B *LpRRs*	*LpRR2*	Fold change	0.65	0.19	0.50
		SD	0.097	0.02	0.07
	LpRR10	Fold change	0.598	0.45	0.55
		SD	0.16	0.28	0.17
	LpRR12a-2138	Fold change	0.51	0.14	0.18
		SD	0.17	0.02	0.00
	LpRR12a-2183	Fold change	0.57	0.15	0.51
		SD	0.081	0.01	0.02
	LpRR12b	Fold change	0.649	0.11	0.27
		SD	0.13	0.01	0.34

Table 4.2. Relative expression of cytokinin response regulator (*RR*) gene family members in the root of perennial ryegrass after treatment with water and 0.1% DMSO (control) immediately before (d0) or at one (d1) or seven (d7) days after N treatment.

	***RR* family members**	**Expression**	**d0**	**d1**	**d7**
Type-A *LpRRs*	*LpRR1*	Fold change	0.063	0.313	0.116
		SD	0.002	0.029	0.013
	LpRR3	Fold change	0.091	0.122	0.016
		SD	0.047	0.029	0.010
	LpRR6	Fold change	0.081	0.181	0.114
		SD	0.005	0.023	0.038
	LpRR9	Fold change	0.021	0.240	0.115
		SD	0.003	0.014	0.014
Type-B *LpRRs*	*LpRR2*	Fold change	0.937	0.559	0.368
		SD	0.062	0.096	0.026
	LpRR10	Fold change	0.639	0.297	0.580
		SD	0.113	0.035	0.037
	LpRR12a-2138	Fold change	0.825	0.328	0.352
		SD	0.168	0.222	0.131
	LpRR12a-2183	Fold change	0.906	0.609	0.273
		SD	0.086	0.067	0.086
	LpRR12b	Fold change	0.507	0.588	0.414
		SD	0.054	0.130	0.028

Data obtained from plants treated with 6-BAP or PI-55 were summarised in a heat map representing the negative (blue) or positive (red) fold changes of expression of *LpRR* gene family members. Numbers indicated in each cell correspond to relative fold change in expression and were calculated for each *LpRR* gene family members in plants treated with 6-BAP or PI-55 and 0.1% DMSO relative to control plants (Fig 4.8).

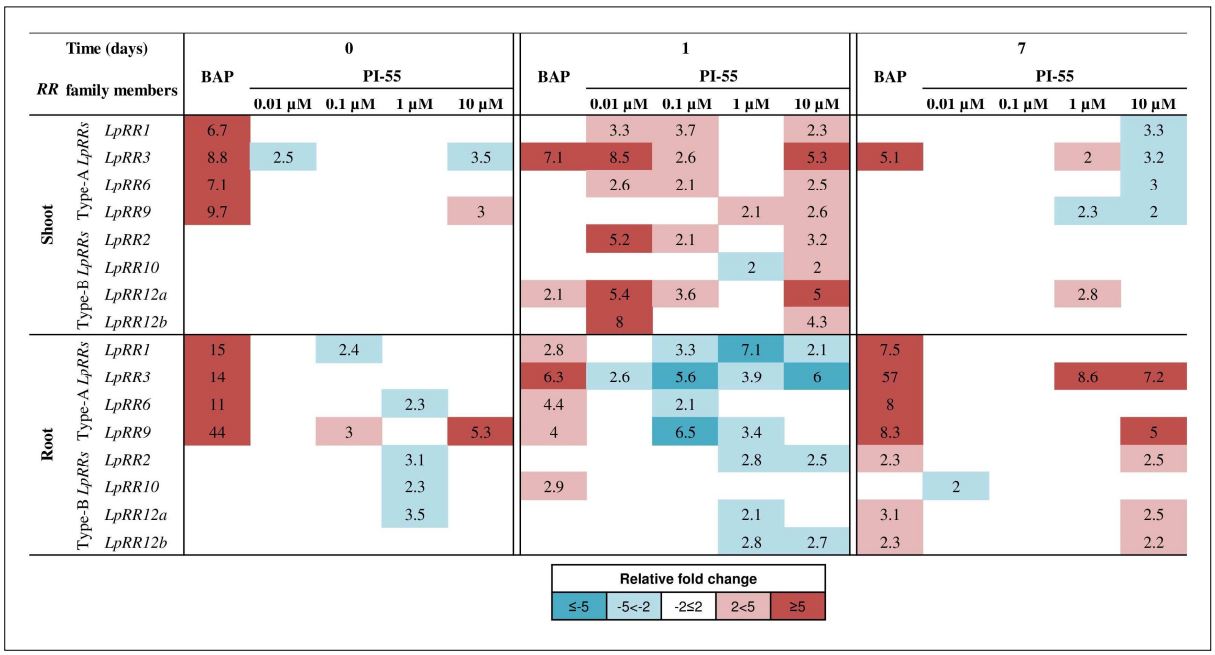


Figure 4.8. Relative expression of cytokinin response regulator (*RR*) gene family members in root and shoot tissues of perennial ryegrass. Negative (blue) or positive (red) values were fold changes relative to the control plants treated with 0.1% DMSO and water instead of 6-BAP or PI-55, and calculated using reference genes *GAPDH* and *EF* as internal controls of relative gene expression per sample. Numbers indicated in each cell correspond to relative fold change in expression and were calculated for each *LpRR* gene family members by dividing the fold change expression level of the treated plant with the fold change values obtained for the control plant for each time point. Plants were treated with 1 µM of 6-BAP or with various concentrations of PI-55 one day before treatment with 5 mM KNO_3 (d0). Control plants were left untreated. 0 = immediately before N treatment, and 1, 7 for one, and seven days after N addition respectively. Each value represents the mean of three technical replicates using a cDNA from a pool of five plants as the RT-qPCR template.

The trends of *LpRR* expression observed in Tables 4.1 and 4.2 are consistent with the trends presented in Figure 2.6 (see Chapter 2 for details). Treatment with 1 µM 6-BAP was associated with an initial up-regulation of all Type-A *LpRR*s at d0 relative to control treated plants (Fig. 4.8). Following N treatment, only Type-A *LpRR3* remained up-regulated in the shoot, while the root Type-A *LpRR* expression was transiently down-regulated after 6-BAP treatment at d1 relative to the other time points. Notably, a progressive up-regulation of Type-B *LpRR* family members was observed over time in the root treated with 6-BAP.

At d0, PI-55 had little impact on *LpRR* gene expression profiling relative to d0 control plants and most changes associated with PI-55 treatments were measured at d1 (Fig. 4.8). In particular, an overall up-regulation of *LpRR* family members was detected in the shoot of PI-55 treated plants, whereas a down-regulation correlated with the PI-55 concentration was recorded in the root at d1. Indeed, increased concentrations of PI-55 were generally associated with the down-regulation of more Type-B *LpRR* family members in the root at d1. Overall, by seven days after N treatment, the profiling of the *LpRR* members was not affected by PI-55 treatments at 0.01 µM and 0.1 µM concentrations, whereas increased change in expression were observed at higher PI-55 concentrations relative to control plants. Opposite trends between tissues were noted at d7 under 10 µM PI-55: down-regulation of all Type-A *LpRRs* in the shoot and up-regulation of most *LpRRs* members in the root. More complex trends were noted under 1 µM PI-55 relative to the other PI-55 treatments over time.

4. 4 Discussion

4.4.1 Exogenous CPPU application does not increase the physiological responses of *Lolium perenne* plants to nitrate treatment and defoliation

Commercial field applications of cytokinin-like compounds on vegetables, fruit trees, and cotton has traditionally been very successfully used to increase seed size, seed number, and harvest index, defined as the ratio of harvested grain to total shoot dry matter (Sinclair 1998, Jameson and Song 2016, Koprna et al. 2016). In addition, exogenous cytokinin treatments promoted tiller number and tiller bud elongation in rice (Zahir et al. 2001), wheat (Langer et al. 1973), barley (Suge and Iwamura 1993), and oats (Harrison and Kaufman 1980). However, field applications of cytokinins to cereals has had a limited success (Jameson and Song 2016). To date, little is known about the use of exogenous cytokinin applications as a biotechnological tool to improve crop biomass of pasture species such as *Lolium perenne*.

The present study does not suggest any practical advantage of applying the cytokinin-like compound CPPU to increase perennial ryegrass plants NUE and regrowth following N treatment and defoliation, as measured by the dry weight of regrown leaf (Fig. 4.2), tiller number (Fig 4.3A), leaf number (Fig. 4.3B), and maximum photosynthetic capacity A_{max} (Fig. 4.4A) over a 28 day period. CPPU treatments were associated with a decrease in leaf regrowth rate one day after defoliation (Fig. 4.3C) and decrease in stomatal conductance g_s,

although only at 28 days after N addition and cut (Fig. 4.4B). The lack of positive growth responses following defoliation is opposite to physiological and growth measurements obtained in Wang et al. (2013) study. Indeed, the author found that exogenous cytokinin application resulted in a higher content of the endogenous cytokinin forms zeatin, zeatin riboside, isopentenyladenine, and isopentenyladenosine, which led to a greater biomass of newly grown leaves relative to the ryegrass plants not sprayed with cytokinin (Wang et al. 2013). The differences in regrowth response following N treatments and defoliation may have been influenced by a number of experimental differences discussed hereafter.

The present CPPU experiment analysed five-month old perennial ryegrass plants and Wang et al. (2013) study was performed on 11-week-old Italian ryegrass (*Lolium multiflorum*) plants. It is possible that the older plants were growing less actively and were less responsive to cytokinin treatments than younger plants. In addition, Wang et al. (2013) watered the plants with a modified Hoagland solution containing 5 mM of KNO_3, which may have resulted in plants metabolically more active than the plants in the present study. Consistently with findings in barley and rice, contrasting cytokinin responses may be associated with different stages of tiller development (Yang et al. 2003) and with different cultivars (Hosseini et al. 2008).

Cytokinin activity is a common property of two distinct classes of chemical compounds: N^6-substituted adenine derivatives, such as zeatin, isopentenyladenine, 6-BAP, and substituted phenylureas, such as CPPU (Capelle et al. 1983). The effect of exogenous cytokinin treatment on ryegrass regrowth response to N-addition was investigated by application of the naturally occurring cytokinin 6-BAP in Wang et al. (2013) study, whereas a the synthetic cytokinin-like CPPU compound was used in the present study. Laloue and Fox (1989) showed that CPPU can act by inhibiting the activity of cytokinin oxidase, the enzyme responsible for cytokinin degradation into adenine and adenosine (Laloue and Fox 1989). This suggests a distinct endogenous molecular mode of action of CPPU from the 6-BAP cytokinin. However, more recent work with diphenyl ureas suggest that they can also bind directly and strongly activate all three *A. thaliana* cytokinin receptors AHK2, AHK3, and CRE1/AHK4 (Spíchal et al. 2004, Stolz et al. 2011, Nisler et al. 2016). It can be hypothesised that the physiological underpinnings associated with exogenous CPPU and 6-BAP treatments might be controlled by distinct and/ or overlapping complex molecular mechanisms due to contrasting endogenous cytokinin forms, their relative transport, and metabolism.

Although repeated cycles of cytokinin application provided an interesting framework to investigate the physiological responses of ryegrass plants to exogenous cytokinin application, the growth responses recorded by Wang et al. (2013) would not be expected in a field situation. Indeed, ambivalent consequences of cytokinin field applications are associated with a limited timing of cytokinin application in regards to cell division phase, and are due to diverse source and sink strength and developmental stages of the plants present in field trials (Yang et al. 2003, Jameson and Song 2016). It is likely that different techniques of cytokinin application may influence the cytokinin-induced growth responses. Indeed, foliar application of cytokinins is a powerful and convenient method for field application, although it can be loss due to rain, run-off from hydrophobic surfaces, and rapid drying of the spray solution which limits the penetration of the solution through the leaf surface (Nair et al. 2010). Interestingly, CPPU application in kiwifruit resulted in a decrease in the endogenous cytokinin concentration, which may have been caused by a feedback inhibition following activation of the cytokinin signalling pathway (Lewis et al. 1996). Similarly, hydroponic treatments with 6-BAP in the present study resulted in an overall down-regulation of the Type-A *LpRRs* and progressive up-regulated of the Type-B *LpRRs* relative to control plants at d1 and d7 after N treatment (Fig. 4.8). The Type-B *LpRRs* are considered to act as direct negative transcriptional regulators of Type-A *LpRRs*, and regulators of other target genes (Dortay et al. 2006, Jain et al. 2006, Sakakibara 2006). Therefore, *LpRRs* profiles of expression at d1 and d7 suggest a negative feedback mechanism dampening the cytokinin signalling associated with the response over time to an exogenous cytokinin treatment and/ or to the N-induced cytokinin response (d0) (Fig. 4.8).

4.4.2 PI-55 inhibition of the cytokinin signalling pathway in the root is not associated with physiological consequences

To date, the direct involvement of cytokinin signalling in diverse developmental processes has been identified using *A. thaliana* mutants and transgenic of cytokinin synthesis (Takei et al. 2004), enhanced degradation (Werner et al. 2003), cytokinin receptors (Riefler et al. 2006) or proteins involved in the cytokinin signalling pathway (Hutchison et al. 2006). The use of anticytokinin chemicals would be a very useful alternative tool to genetic studies in non-model species, such as *L. perenne*, for which collections of mutants aren't available and knowledge on the genome sequences limited (Jones et al. 2001, Sawbridge et al. 2003,

Velmurugan et al. 2016). The present study is the first report of the use of an inhibitor of cytokinin receptors, PI-55, to investigate the relative importance of the long-distance cytokinin signalling in the N response of perennial ryegrass. Under our experimental conditions, perturbations of cytokinin signalling by PI-55 treatment did not significantly influence the growth response of perennial ryegrass plants following N addition (Figs. 4.6, 4.7). This highlights the importance of the integration of the NO_3^- specific long-distance signalling with other signals such as light and sugar for a synchronised development of the plant.

In the present study, treatment with PI-55 resulted in changes in expression of the cytokinin response regulators relative to the control, with most changes detected one day after NO_3^- addition (Fig. 4.8). Hence, PI-55 was able to perturb the cytokinin-mediated N signalling pathway in perennial ryegrass (Fig. 4.8). Increased concentrations of PI-55 were associated with greater changes in the Type-B *LpRR* family members relative to control plants in the root at d1 (Fig. 4.8). This is consistent with a PI-55 dose-dependent regulation of the expression of the cytokinin response described by Spíchal et al. (2009) in transgenic *A. thaliana* seedlings transformed with the *ARR5:GUS* reporter gene. The greatest changes in cytokinin signalling relative to control plants were observed by treatment with 10 µM PI-55, suggesting that this would be the optimum concentration for further investigation (Fig. 4.8).

4.4.3 Putative agonist effect of PI-55 in the shoot and tissue-specific regulation of the cytokinin signalling pathway by PI-55 following N-treatment

To be considered as antagonist of cytokinin receptors, a compound must have structural similarity with the active cytokinin but a minimum cytokinin agonist activity for competitive fixation on the cytokinin receptor sites (Spichal et al. 2010). Therefore, treatment with PI-55 in perennial ryegrass would be expected to bind the cytokinin receptors with minimum activation of the cytokinin signalling pathway, resulting in an overall lower expression of the *LpRRs* gene family members relative to control plants not treated with PI-55. The results obtained in the present study indicate a down-regulation in *LpRR*s expression level in the root at d1, whereas an overall induction was recorded in the shoot at d1 (Fig. 4.8).

PI-55 was initially identified as a competitive antagonist of *t*Z binding to the *A. thaliana* cytokinin receptors CRE1/AHK4 and AHK3 (Spíchal et al. 2009). In addition, Spíchal et al.

(2009) noted that PI-55 may act as a weak agonist of the *A. thaliana* receptors AHK3 and AHK2 (Spíchal et al. 2009). Over the past decade, the homologous cytokinin receptor genes to the *A. thaliana* CRE1/AHK4, AHK3, and AHK2 have been identified and characterised in numerous other plant species, such as *Populus trichocarpa* (Nieminen et al. 2008), *Oryza sativa* (Ito and Kurata 2006, Du et al. 2007), and *Zea mays* (Yonekura-Sakakibara et al. 2004). Ueguchi et al. (2001) recorded tissue-specific expression of the cytokinin receptors: *CRE1/AHK4* was primarily detected in the root tissue and weakly in the stem, whereas *AHK2* was expressed in both the root and leaf although at weak levels relative to the other *AHKs* levels of expression, and *AHK3* transcript was detected in the root, leaf, and stem (Ueguchi et al. 2001).

Yonekura-Sakakibara et al. (2004) found that *Z. mays* cytokinin receptors can differ in ligand preference from the *A. thaliana* receptors. Namely, a stronger response to isopentenyladenine (iP) was noted in the *A.thaliana CRE1/AHK4* cytokinin receptors and *Z. mays* orthologue ZmHK1 relative to *trans*-zeatin (*t*Z), whereas *AHK3*, and its orthologue *ZmHK2* the opposite trend was observed (Yonekura-Sakakibara et al. 2004, Heyl et al. 2012). In addition, the cytokinin response regulator *ZmRR1* was found to be induced by *cis*-zeatin (*c*Z) as well as by *t*Z in cultured *Z. mays* cells (Yonekura-Sakakibara et al. 2004). Substantial amount of *c*Z were detected in maize and other species from the Poaceas family, such as *L. perenne* (Gajdošová et al. 2011), whereas this cytokinin form is found at low levels and only weakly active in *A. thaliana* (Yonekura-Sakakibara et al. 2004).

Yonekura-Sakakibara et al. (2004) results strongly suggest that *Z. mays* cytokinin receptors differ in ligand specificity from the *A. thaliana* receptors. Considering the greater proportion of *c*Z in perennial ryegrass when compared with *A. thaliana*, it is possible that PI-55 action had a lower anticytokinin activity by competitive binding to cytokinin receptors in *L. perenne*. Furthermore, the tissue-specific regulation of the cytokinin signalling pathway by PI-55 observed in the present study could imply that PI-55 may act mainly at the site of application, or have acted specifically on the root cytokinin receptors, or possibly was not efficient in inhibiting the cytokinin receptors and associated cytokinin signalling pathway in the shoots of perennial ryegrass plants.

4. 5 Conclusion

A coordinated whole-scale physiological response to N involves long-distance signalling associated with a specific and a cytokinin-mediated N signalling pathway in plants (Sakakibara 2003). A systems biology approach revealed that NO_3^- responsive genes can be regulated by N signalling, hormonal signalling or by a N/ hormone cross-control (Nero et al. 2009). The cytokinin-mediated control of nutrient pathways has a broad effect on the metabolism of the plant as a whole (Sakakibara et al. 2006*b*). Furthermore, Nero et al. (2009) found that hormone signals can enhance the responsiveness of a wide variety of genes and metabolic pathways regulated by NO_3^-, and important for the control of plant development (Nero et al. 2009).

This chapter highlights the lack of understanding of the cytokinin-mediated N signalling pathway and its associated control over the physiological responses to N addition in the non-model species perennial ryegrass. The contrasting results observed between the literature and the CPPU results presented in this chapter indicates a need to develop a better understanding of the molecular events taking place over time following CPPU application, if we are to target changes in content of specific endogenous cytokinins as a tool to ultimately improve NUE and regrowth of perennial ryegrass plants. The PI-55 experiment resulted in tissue-specific regulation of the cytokinin signalling pathway, which suggests species-specific differences in cytokinin forms, ligand-specificity and site of expression of the cytokinin receptors. The PI-55 down-regulation of the cytokinin signalling pathway observed in the root suggests that 10 µM of PI-55 could be used for further investigation of the anticytokinin effect of PI-55 in perennial ryegrass roots.

Chapter 5

General discussion and conclusions

Contents

5. 1 Conceptual model of principal findings

This research was undertaken to investigate the physiological underpinnings of nitrate (NO_3^-) assimilation in order to improve nitrogen (N) use efficiency (NUE) in perennial ryegrass and limit environmental side-effects associated with the excessive use of N fertilisers. Of particular interest was the response of perennial ryegrass plants over the seven days following NO_3^- treatment (Chapter 2), defoliation (Chapter 3), and exogenous application of a cytokinin-like compound and cytokinin antagonist (Chapter 4). This project includes spatiotemporal studies of the effect of N status and carbon (C) remobilisation on NO_3^- assimilation and associated cytokinin signalling in leaf, sheath, and root of perennial ryegrass plants using physiological measurements, un-targeted and targeted metabolomics, and gene expression analysis of cytokinin response regulators (*LpRRs*).

Figure 5.1 represents a conceptual model highlighting key steps (numbers with purple background) in NO_3^- use of perennial ryegrass and the principal findings reported in Chapters 2, 3, and 4, and from the literature (Chapter 1).

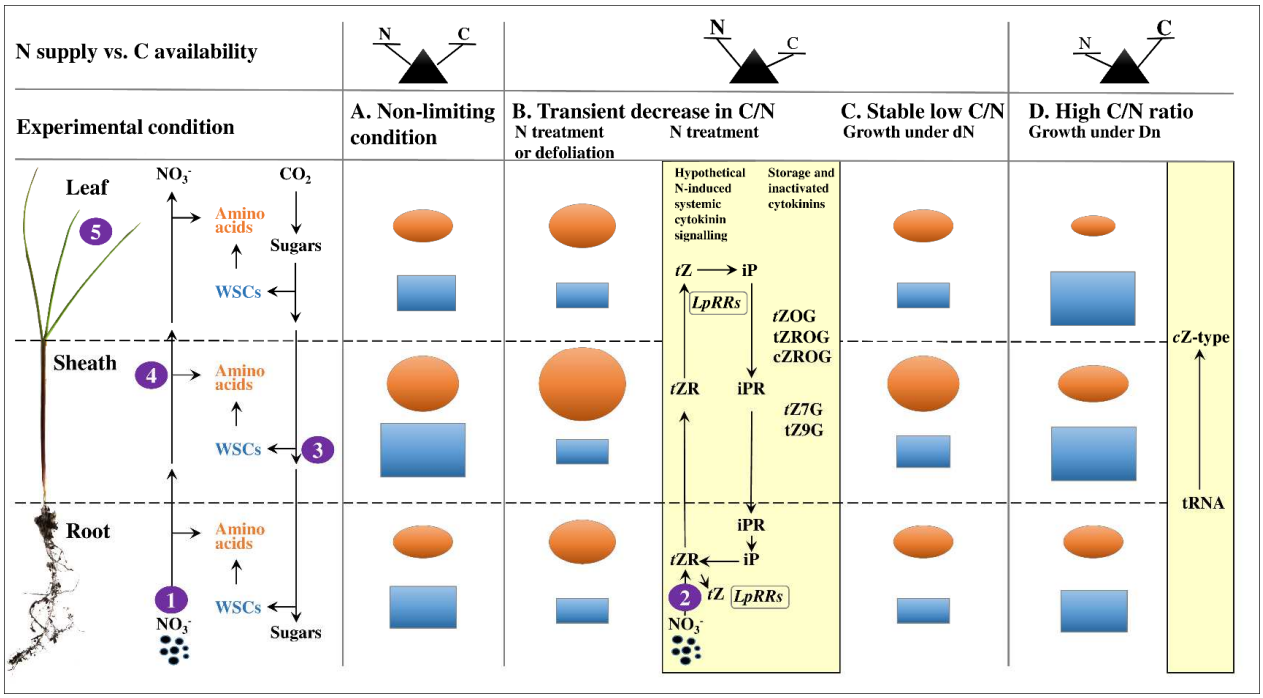


Figure 5.1. Relative proportions of the water-soluble carbohydrates (WSCs) (blue rectangles) and amino acids (orange ovals) under contrasting nitrogen (N) supply and carbon (C) availability, and associated experimental conditions: non-limiting growth condition (A), transient decrease in C/N ratio (B), steady-state low C/N (C), and high C/N ratio (D). Growth under dN and Dn correspond to plants exposed to short days (8 h light: 16 h dark, d) or continuous light (D), and watered with modified N-free Hoagland medium containing either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source. The size of the rectangles and ovals is proportional to their relative abundance. Numbers with purple background correspond to key steps in NO_3^- use and associated findings identified from the present study. Details for each number can be found in the text.

1. Nitrate treatment to perennial ryegrass plants resulted in N uptake, initial N accumulation in the root, and subsequent more gradual N accumulation in the shoot relative to treatment with KCl (Figs. 2.2A and 5.1B).

2. The present study is the first report of gene expression analysis of the cytokinin response regulators *LpRRs* in perennial ryegrass. The relative changes in *LpRR* expression suggested an early activation of the cytokinin signalling pathway and subsequent negative feedback regulation dampening the cytokinin response within 24 h of N addition and/or exogenous 6-BAP treatment (Figs. 2.6, 4.8, Tables 4.1 and 4.2). Active and transported cytokinin forms changed within 24 h following an addition of NO_3^- to N-deficient plants or C stress induced by defoliation, which could suggest a long-distance cytokinin signalling between perennial ryegrass roots and shoots in response to transient decrease in C/N ratio (Figs. 2.5, 3.5 and 5.1B). A subsequent increase in the storage and inactivated cytokinin forms could indicate an activation of cytokinin homeostasis at seven days after N addition (Fig. 2.5). Growth under steady-state N deficiency was hypothesised to enhance tRNA turnover but not translocation, and to result in high content of the *cis*-zeatin (*c*Z)-type cytokinins (Figs. 2.5B, 3.5 and 5.1D).

3. An inverse relationship was found between N supply and WSCs content (Figs. 2.3, 3.2, 3.3, 5.1). Indeed, N-deficient plants accumulated WSCs (Figs. 2.3, 3.2B and 5.1D), whereas N supply, defoliation or growth under dN resulted in remobilisation of the WSCs stores (Figs 2.3, 3.2B, 3.3 and 5.1B, C) relative to plants under non-limiting conditions (Figs. 3.2B, 5.1A). Plants with a decreased ratio of C availability over N supply could re-establish the internal C/N balance by an initial remobilisation of the low-molecular weight (LMW) WSCs

within 24 h and by subsequent breakdown of the high-molecular weight (HMW) WSCs for large C remobilisation in the shoot (Figs. 2.3, 3.3 and 5.1B, C), whereas the root WSCs became progressively depleted over the seven-day period following defoliation (Figs. 2.3B, 3.3 and 5.1B).)

4. An efficient NO_3^- assimilation relies on a coordinated C remobilisation with N supply for conversion into amino acids, tricarboxylic acid intermediates, and other complex N-containing molecules (Figs. 2.4, 3.4 and 5.1). Defoliation resulted in a marked amino acid synthesis and storage within a day, and a subsequent negative feedback reduced the defoliation-induced amino acid synthesis by seven days, with tissue-specificities and timing of response dependent on N supply and C availability (Figs 3.4 and 5.1B).

5. The efficiency of N use by the plant is a critical component of forage production and plant regrowth in response to defoliation (Wilkins et al. 1997, Raun and Johnson 1999). A greater N availability promoted tiller and leaf growth under long days relative to plants exposed to a lower N availability (Figs. 2.2C, D, 3.1 and 5.1). Exogenous applications of CPPU, a cytokinin-like compound, and of a cytokinin receptor antagonist, referred to as PI-55, did not suggest the direct control of cytokinins over leaf and tiller number (Figs. 4.3, 4.5 and 4.7), leaf regrowth rate (Figs. 4.3 and 4.5), regrown leaf biomass (Figs. 4.2, 4.5 and 4.6), and photosynthesis (Fig. 4.4) following N treatment and/or defoliation under the experimental conditions of the present study.

5. 2 General discussion

This project was funded by Ballance Agri-Nutrients Limited New Zealand in order to obtain recommendations regarding the benefits of applying N fertiliser combined with cytokinins to improve NUE of perennial ryegrass plants and to develop an understanding of the external and internal regulators of N use in a pasture crop.

Cytokinin synthesis and signalling are required throughout the plant life cycle for its development and for nutrient use (Mok and Mok 2001, Sakakibara et al. 2006*b*, Werner and Schmülling 2009). Several lines of evidence indicate that exogenous application of various plant hormones can result in positive growth effects for a number of plant species (Calvo et

al. 2014). To date, very little is known about the relative importance of cytokinins for NUE in perennial ryegrass (Wang et al. 2012, Wang et al. 2013, Zaman et al. 2016). The present study revealed that exogenous cytokinin applications did not increase the physiological response of perennial ryegrass plants to NO_3^- treatment and defoliation (Figs. 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7). A number of molecular mechanisms associated with cytokinin metabolism and signalling were identified in response to perturbations of the C/N ratio, although treatments with CPPU and PI-55 highlighted a lack of understanding of the N mediated cytokinin signalling (Fig. 4.8). In light of the present results and information taken from the literature, which indicated an inhibitory effect of cytokinin on root growth (Ioio et al. 2007) and limitations associated with field applications of plant hormones to certain crops (Jameson and Song 2016), the data in the present study do not support a recommendation for the development of a cytokinin based N fertiliser to improve perennial ryegrass NUE.

Perennial ryegrass is a temperate forage grass usually receiving repeated N fertiliser application after bovine grazing (Miller et al. 2001, Wilkins and Humphreys 2003). The efficiency of N assimilation in response to defoliation is a critical component of plant regrowth and biomass production (Norton et al. 2015). The present study characterised a coordinated remobilisation of WSCs for synthesis of amino acids and subsequent biomass production in perennial ryegrass plants following NO_3^- addition and/or defoliation (Figs. 2.2, 2.3, 2.4, 3.1, 3.2, 3.3, 3.4 and 5.1). Taken together, these results lead to the recommendation that a grazing event and N fertiliser application should be separated in time by several days to allow a recovery of some of the WSCs reserves required for efficient N use and regrowth of perennial ryegrass plants.

Nitrogen use efficiency can be defined as the plant biomass produced per unit of applied N (Good et al. 2004, Xu et al. 2012). However, this definition does not include an estimate of the N content per leaf dry matter. Nitrogen is mainly stored in the plant in the form of proteins, which are of important nutritional value for animal consumption (Kingston-Smith et al. 2006). Hence, at fixed amount of N input, an increase in biomass production would be considered as an increase in NUE, although it would be correlated with dilution of nutritional components (Monaghan et al. 2001, Hawkesford and Barraclough 2011). The present study reported important changes in N containing free amino acids and fructan content associated with physiological responses following N treatments (Figs. 2.2, 2.3, 2.4, 3.1, 3.2, 3.3, 3.4 and 5.1). Therefore, a relevant estimate of NUE for perennial ryegrass plants may include N

uptake efficiency, leaf biomass and percentage of several nutritional compounds, such as proteins, fibres, sugars, and fructans per unit of leaf dry weight. The present study also led to the consideration that measurements of NUE over several time points provides a better information regarding the physiological and molecular mechanisms controlling NUE.

Nitrogen assimilation is initiated by the production of Gln, Glu, Asn, and Asp, and subsequent conversion into other amino acids (Miflin and Lea 1977). Several enzymes and isoenzymes are involved in the amino acid biosynthesis and are differentially regulated by environmental factors, developmental stages, and metabolism (Lea and Forde 1994). In addition to their role as important N carriers in plants, the amino acids can serve as precursors of specific metabolic pathways (Lam et al. 1996). In the present study, contrasting profiles of individual amino acid form and content between tissues support tissue-specific regulation of the enzymes associated with biosynthesis, degradation, and transport of amino acid (Figs. 2.4 and 3.4). Therefore, a more developed understanding of the amino acid metabolism could lead to improvement in protein content in the leaf and in NUE by using breeding and genetic techniques (Shewry Jones 2005, Lea and Azevedo 2007).

There are significant gaps in the current understanding of fructan metabolism and endogenous regulation of fructan content (Turner et al. 2006, Harrison 2012). Results obtained from the N treatment (Fig. 2.3) and defoliation experiments (Fig. 3.3) revealed notable differences in overall profiling of the relative abundance of WSCs across degree of polymerisation (DP). Indeed, the overall trend within the N-deprived plants was that the higher the DP, the more likely the WSCs was to be found in the shoot rather than in the root (Fig 2.3F). In addition, the relative contribution in WSCs increased from DP7 to DP14 and decreased for higher DPs (Fig. 2.3A, B). This DP profiling was less obvious in plants watered with N-free Hoagland medium modified with either high (5 mM, N) or low (50 µM, n) NO_3^- as sole N source for two weeks (Fig. 3.3). Fructans in grasses are highly diverse in chain length, weight, and linkage type between monomeric sugar units (Vijn et al. 1997, Chalmers et al. 2005, Lothier et al. 2007). Temperate grasses accumulate complex mixture of fructans, such as inulin serie, inulin neoseries, and levan neoseries in *Lolium perenne* (Pavis et al. 2001). Therefore, the differences in fructan profiling observed in the present study may reflect structural as well as functional differences in WSCs, which may be important for perennial ryegrass NUE.

The data presented in this thesis highlights the importance of a mechanistic coordination between leaf, sheath, and root tissues for assimilation of inorganic N onto C skeletons. Distinct and complementary functions were characterised across the three tissue types, irrespective of the N supply and C availability. Nitrogen taken up by the root was mainly stored in the leaf (Figs. 2.2, 3.2 and 5.1) (Moser et al. 1982). The sheath tissue was the main site of storage of C (Figs. 2.2, 2.3, 3.2, 3.3 and 5.1) (Prud'homme et al. 1992) and site of amino acid accumulation and/or synthesis (Figs. 2.4, 3.2, 3.4 and 5.1) (Bigot et al. 1991). This suggests an important role of the sheath in the grazing tolerance of perennial ryegrass plants due to storage of important compounds and particularly of the long-term storage carbohydrate.

A decrease in C/N ratio induced changes in cytokinin metabolism, which preceded or were concomitant with the WSCs remobilisation for amino acid synthesis (Figs. 2.3, 2.5, 3.3 and 3.5), although the response to N treatment and to defoliation were associated with distinct cytokinin profiles (Figs. 2.5 and 3.5). This leads to the hypothesis that a cytokinin-signalling pathway is involved in the coordination of WSC breakdown with N use across perennial ryegrass tissues.

Figure 5.2 is a schematic representation of the relative importance of the N induced cytokinin-dependent and cytokinin-independent pathways regulating shoot growth. The present model differs from the previous models of Dodd and Beveridge (2006) and Wang et al. (2013) in that it includes results obtained in this study associated with key discussion points (letters with purple background), which support or contrast with the previous models.

Consistent with Wang et al. (2013) model, the results obtained in the present study revealed that defoliation was associated with a decrease in some organic substances in the root, such as the WSCs (Figs. 3.3 and 5.2A), although further correlation between WSC content, NO_3^- uptake and N delivery to the shoot was not investigated.

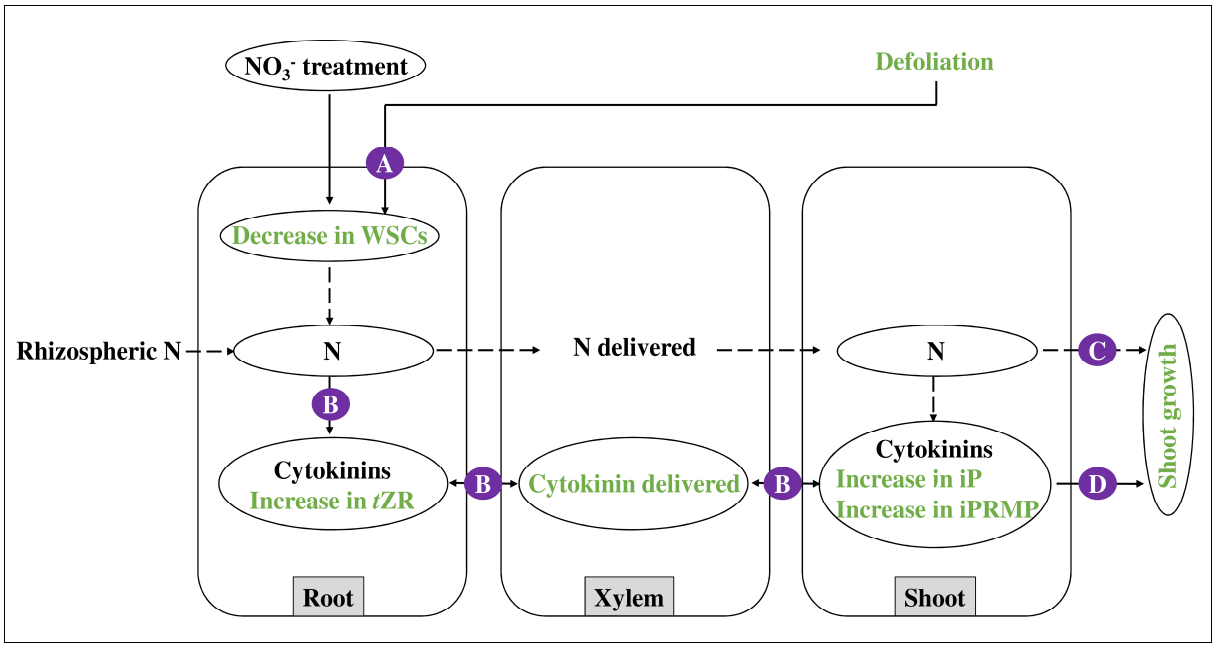


Figure 5.2. Putative schemes linking changes in water-soluble carbohydrates (WSCs), nitrogen (N) and cytokinin content in the root, xylem, and shoot of perennial ryegrass plants following defoliation (green font) or NO_3^- treatment (oval frame). A dashed arrow indicates a hypothetical relationship and a solid arrow represents a strong relationship identified from the results of the present study. Letters with purple background correspond to key discussion points from the comparison between the models from Dodd and Beveridge (2006), Wang et al. (2013), and from the findings identified in the present study. Details for each letter can be found in the text.

Nitrate treatment of N-deficient plants resulted in an extensive accumulation in the active and transported cytokinin forms (Fig. 2.5). Based on Kudo et al. (2010) model, NO_3^- treatment could induce the synthesis of *t*ZR, which would act as an acropetal messenger regulating shoot growth, whereas the iP-type cytokinins could act as systemic or basipetal messenger (Figs. 5.1B and 5.2B). As early as 8 h after defoliation, increased levels of *t*ZR cytokinins were detected in the root of perennial ryegrass plants (Fig. 3.5), whereas an accumulation of the iP and iPRMP cytokinin forms was recorded in the sheath, which suggested long-distance root to sheath and/or sheath to root cytokinin signalling in response to defoliation (Figs. 3.5 and 5.2B). Therefore, the data obtained in the present study are consistent with Dodd (2005) report of a link between changes in N concentration to the root and changes in cytokinin in the root, xylem, and leaf tissues (Fig. 5.2B). This contrasts with Wang et al. (2013) model in

which a decrease in root organic substances had no effect on the cytokinin forms in the xylem and in the leaf (Fig 5.2B).

In addition, Wang et al. (2013) suggested that leaf NO_3^- content had no effect on the biomass of the regrown leaves, whereas the data presented in the present study indicate that an increase in N content preceded an increase in leaf biomass, although a causal role was not established in the present study and such measurements were not performed on plants under frequent defoliation (Figs. 2.2 and 5.2C). Leaf regrowth was not influenced by exogenous foliar application of CPPU or PI-55 (Figs. 4.2, 4.3 and 4.5), which does not tend to support Wang et al. (2013) model describing the negative effects of a decline in leaf cytokinin concentration on perennial ryegrass biomass of regrown leaves (Fig. 5.2D).

Dodd and Beveridge (2006) includes rhizospheric N in their model. Under field conditions, plant nutrient use is highly dependent on organic and on inorganic N uptake (Glass et al. 2002, Tabata et al. 2014), cross-talk with other nutrients/signals such as phosphorous (Yasuda et al. 2014), potassium (Amtmann and Armengaud 2009), and other plant hormones (Forde 2002). In addition, N metabolism is affected by the interaction of the plant with other organisms, such as fungal endophytes, free-living and endophytic N_2 fixing bacteria (Andrews et al. 2003, Rasmussen et al. 2007). Nitrogen use under field conditions is closely associated with water availability, which can have a direct effect on NUE (Akmal and Janssens 2004), and on the endophyte profiling of perennial ryegrass plants (Hesse et al. 2005). Although measurements under hydroponic conditions offered a precisely controlled environment for molecular analysis, a number of important variables regulating N assimilation should be taken into considerations while transferring the knowledge to field conditions.

The results obtained in the present study have raised a number of important questions, which needs further investigation:

1. Field studies are required to verify the relationship between WSCs remobilisation and efficiency of N assimilation observed under controlled conditions in the present study.

2. The spatiotemporal analysis performed in the present study was obtained using *L. perenne* L. cv. “Grasslands Nui”. Screening of wide populations of perennial ryegrass could

potentially lead to the selection of more N efficient cultivars. Measurements should include N uptake efficiency, leaf biomass, WSC profiles, content of several other nutritional compounds and root system architecture, before and after defoliation.

3. Cycles of N addition and defoliation resulted in a decrease in biomass production (Fig. 4.5A). It was hypothesised that the WSC availability might be limiting N assimilation during regrowth (Figs. 2.3, 3.3 and 4.5A). Field studies should be conducted to define the best timing of N application following defoliation, in terms of WSC availability. An interesting investigation would include estimates of the content and nature of the various branching forms of the WSCs, as well as the expression profiling of the genes involved in fructan biosynthesis and degradation, across seasons, for a given cultivar of perennial ryegrass. This would develop a better understanding of the relative importance of the individual WSC forms for N assimilation and ultimately for biomass production, which would likely provide useful information for breeders.

4. Nitrogen assimilation can result from the assimilation of NO_3^- directly taken up or from N recycling from endogenous N-containing molecules that had previously accumulated (Ourry et al. 1990). Isotopic measurements of N flux would enable the estimation of the relative proportion of remobilised N used for amino acid synthesis.

5. The present work has mainly investigated N assimilation, although NO_3^- uptake is another important component of NUE. Of particular interest, the regulation of the high- and low-affinity NO_3^- uptake transport systems by the remobilised WSCs and associated cytokinins should be investigated in response to changing C/N ratio. Measurements of root NO_3^- uptake and NO_3^- delivery rate by isotopic labelling, as well as cytokinin content in the xylem following N treatment and/or non-frequent defoliation could add to the model linking WSCs, N and cytokinins (Fig. 5.2).

6. Rhizospheric N in natural ecosystems corresponds to organic and inorganic N (Harrison et al. 2007, Näsholm et al. 2009). Treatments of perennial ryegrass with urea, NH_4NO_3, NH_4^+ or NO_3^+ would develop a better understanding of the specificity of N signalling and/or N dependant cytokinin signalling.

7. Most molecular data on NUE comes from the dicotyledonous model plant species *Arabidopsis thaliana*, whereas agronomical knowledge has been obtained from cereal species (Girin et al. 2014). Translational research between such species represents a major future challenge with potentially important economic and environmental consequences. Recent discoveries in the genome and transcriptome of perennial ryegrass will facilitate gene expression studies (Studer et al. 2012, Pfeifer et al. 2013, Byrne et al. 2015). Differential transcriptome (RNAseq) analysis of perennial ryegrass leaves, sheath, and roots could help identifying key molecular mechanisms involved in remobilisation of C for NO_3^- assimilation.

8. Key gaps in the current understanding of cytokinin regulation identified in the present study are: the activation and characterisation of the cytokinin receptors, including their localisation and responsiveness to specific cytokinins; the molecular events controlling cytokinin metabolism and signalling in response to C perturbations and the mode of action of PI-55 in perennial ryegrass. Future research studies should investigate these mechanisms.

To conclude, the present study contributed to a better understanding of the effect of N status and C remobilisation on NO_3^- assimilation and associated cytokinin signalling in perennial ryegrass. The results presented in this thesis identified a key challenge: the timing of N application post-grazing, which will need to be addressed in field-simulated or real conditions.

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APPENDIX

Appendix 2. Research article.

Roche J, Love J, Guo Q, Song J, Cao M, Fraser K, Huege J, Jones C, Novák O, Turnbull MH and Jameson PE (2016) Metabolic changes and associated cytokinin signals in response to nitrate assimilation in roots and shoots of Lolium perenne. *Physiologia plantarum* **156**:497-511.

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Metabolic changes and associated cytokinin signals in response to nitrate assimilation in roots and shoots of *Lolium perenne*

Jessica Roche[a], Jonathan Love[a], Qianqian Guo[a], Jiancheng Song[a,b], Mingshu Cao[c], Karl Fraser[c], Jan Huege[c,†], Chris Jones[c,‡], Ondřej Novák[d], Matthew H. Turnbull[a] and Paula E. Jameson[a,*]

[a]School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand
[b]School of Life Sciences, Yantai University, Yantai, 264005, China
[c]AgResearch Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North, New Zealand
[d]Laboratory of Growth Regulators and Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University and Institute of Experimental Botany AS CR, Šlechtitelů 27, 783 71, Olomouc, Czech Republic

Correspondence
*Corresponding author,
e-mail: paula.jameson@canterbury.ac.nz

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The efficiency of inorganic nitrogen (N) assimilation is a critical component of fertilizer use by plants and of forage production in *Lolium perenne*, an important pasture species worldwide. We present a spatiotemporal description of nitrate use efficiency in terms of metabolic responses and carbohydrate remobilization, together with components of cytokinin signal transduction following nitrate addition to N-impoverished plants. Perennial ryegrass (*L. perenne* cv. Grasslands Nui) plants were grown for 10 weeks in unfertilized soil and then treated with nitrate (5 m*M*) hydroponically. Metabolomic analysis by gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry revealed a dynamic interaction between N and carbon metabolism over a week-long time course represented by the relative abundance of amino acids, tricarboxylic acid intermediates and stored water-soluble carbohydrates (WSCs). The initial response to N addition was characterized by a rapid remobilization of carbon stores from the low-molecular weight WSC, along with an increase in N content and assimilation into free amino acids. Subsequently, the shoot became the main source of carbon through remobilization of a large pool of high-molecular weight WSC. Associated quantification of cytokinin levels and expression profiling of putative cytokinin response regulator genes by quantitative reverse transcription polymerase chain reaction support a role for cytokinin in the mediation of the response to N addition in perennial ryegrass. The presence of high levels of *cis*-zeatin-type cytokinins is discussed in the context of hormonal homeostasis under the stress of steady-state N deficiency.

[†]Deceased.
[‡]Present address: Feed and Forage Biosciences, International Livestock Research Institute, PO Box 5689, Addis Ababa, Ethiopia

Abbreviations – cZ, *cis*-zeatin; cZOG, cZ *O*-glucoside; cZR, cZ riboside; cZRMP, cZ ribosyl monophosphate; DP, degrees of polymerization; DW, dry weight; EF, elongation factor 1 alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMW, high-molecular-weight; iP, isopentenyladenine; LMW, low-molecular weight; NUE, nitrogen use efficiency; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RR, response regulator; TCA, tricarboxylic acid; tZ, *trans*-zeatin; tZ7G, tZ-7-glucoside; tZ9G, tZ-9-glucoside; tZOG, tZ *O*-glucoside; WSC, water-soluble carbohydrate;

Introduction

Nitrogen (N) is the most limiting nutrient for plant growth and for crop productivity in many of the world's agricultural areas. In many agricultural systems, forage production relies mainly on fertilizer input supplied as inorganic nitrate (NO_3^-), ammonium (NH_4^+) and urea (Dechorgnat et al. 2011).With only 30–40% of applied N utilized by the plant, intensive use of nitrogenous fertilizer is increasingly causing negative short- and long-term effects on the environment and on farmer economies (Masclaux-Daubresse et al. 2010). The majority of fertilizer is lost through soil denitrification, surface runoff, leaching, volatilization and fluxes of other gaseous emissions to the atmosphere (Dawar et al. 2011 and references therein). Key strategies to consider for more sustainable agriculture are the targeting of N for optimal acquisition by plants, the selection of cultivars that use N more efficiently and the development of a better mechanistic understanding of N use regulation (Hirel et al. 2007). Nitrogen use includes the steps of N uptake, translocation, assimilation and remobilization, and N use efficiency can be defined as total biomass produced per unit of applied N fertilizer (Xu et al. 2012).

Perennial ryegrass (*Lolium perenne*) is a pasture plant used throughout the world because of its ease of establishment and high nutritional value (Humphreys et al. 2010). It plays a central role in the pastoral systems of many countries, and has become the most widely sown perennial forage grass in temperate regions of the United States, China, Japan, UK, Australia, New Zealand, South Africa and South America (Wilkins and Humphreys 2003). Other agronomical advantages of perennial ryegrass include good tolerance to grazing, high digestibility and adequate seed production (Wilkins 1991).

Nitrate use by plants is initiated by NO_3^- transport from the soil to the root through active NO_3^- transporters located in the cell membrane. Subsequent NO_3^- assimilation leads to the biosynthesis of various amino acids, proteins, nucleic acids and other N-derived secondary metabolites. Consequently, N use relies on carbon (C) metabolism to supply C skeletons, ATP for energy and production of reducing equivalents from the breakdown of carbohydrate reserves (Krapp and Traong 2006). Conversely, C metabolism requires enzymatic activity and the coordinated use of N to form cell structural components and to support growth (Zheng 2009).

In grasses and cereals, C is stored primarily as sucrose and fructans (Pollock and Cairns 1991). Fructans are the main storage form of water-soluble carbohydrates (WSCs) in perennial ryegrass, and are a highly accessible energy source (Danckwerts and Gordon 1987). Remobilization of WSCs occurs when photosynthetic C production does not meet the C demand, e.g. to support leaf regrowth after defoliation (Chalmers et al. 2005). WSCs are structurally diverse in their types of linkages and size. They can be composed of linear or branched oligosaccharides of various degrees of polymerization (DP), made up of a fructose residue attached to sucrose (fructan polymers), as well as to other hexoses (Vijn and Smeekens 1999). It has been shown that the content of the WSC stores increases in the shoot with decreasing N nutrition, and treatment with N fertilizer results in remobilization by hydrolysis of WSC reserves in perennial ryegrass (Nowakowski 1962). Conversely, exogenous supply of the mono and disaccharides glucose, sucrose or fructose to the roots of high-N perennial ryegrass stimulated NO_3^- uptake (Louahlia et al. 2008). This suggests a feedback regulation of N use by C availability.

Although the metabolic response of perennial ryegrass to N addition is clearly a complex one, metabolome analysis to investigate N assimilation in perennial ryegrass has not yet been broadly used. Rasmussen et al. (2008) described the metabolic profiles of perennial ryegrass blades when differentially affected by carbohydrate content, high or low N supply for 8 weeks, and fungal endophyte infections. That study showed that under high N conditions, nitrogenous compounds, organic acids and lipids increased in concentration, whereas WSCs, chlorogenic compounds and fiber decreased. Taken together, these results provided insight into N use in perennial ryegrass, but specifically in the blade tissues. However, little is known about the partitioning of NO_3^- assimilation in the root and shoot, especially in the critical days immediately following fertilizer application. Genes involved in NO_3^- metabolism are under control of several local and systemic signals of N supply and demand, which are integrated at the whole plant level (Ruffel et al. 2011).

The cytokinins are a class of plant hormone which control many aspects of plant growth and development (Sakakibara et al. 2006). In *Arabidopsis thaliana*, NO_3^- and cytokinin regulate a variety of genes involved in nitrogen use efficiency (NUE) and C metabolism, and more particularly in processes such as NO_3^- uptake and reduction, ammonia assimilation, amino acid metabolism, glycolysis, organic acid regulation, cell wall expansion and cytokinin metabolism (Sakakibara et al. 2006). Nitrate-mediated cytokinin accumulation appears to be a mechanism conserved among higher plants and has been shown inter alia in *Arabidopsis* (Takei et al. 2002), maize (Sakakibara et al. 1998), rice (Kamada-Nobusada et al. 2013), barley (Samuelson and Larsson 1993), wheat (Garnica et al. 2010) and perennial ryegrass (Wang et al. 2013). The upregulation of specific gene family members coding for isopentenyl

transferase (the rate limiting enzyme in cytokinin biosynthesis) has been shown to occur following NO_3^- addition to N-deficient *Arabidopsis* (*AtIPT3*) (Takei et al. 2004) and rice (*OsIPT4, OsIPT5, OsIPT7* and *OsIPT8*) (Kamada-Nobusada et al. 2013). Microarray analyses in *Arabidopsis* roots have revealed that exogenous application of cytokinin downregulates the expression of NO_3^-, NH_4^+ and amino acid uptake transporter genes, and regulates the NRT1.7 transporter implicated in the remobilization of NO_3^- from root to shoot (Fan et al. 2009). In addition, cytokinin regulates genes important for NO_3^- reduction and N assimilation (Brenner et al. 2005). While NH_4^+ is the main inorganic N source for paddy rice (Kamada-Nobusada et al. 2013), NH_4^+ salts applied alone reduces growth of many plants. However, this negative effect can be ameliorated by application of very low NO_3^- concentrations (Rahayu et al. 2005) or by cytokinin (Shtratnikova et al. 2015), with the conclusion that it is the lack of cytokinin, not NO_3^-, limiting growth in plants supplied with NH_4^+ as sole N source (Shtratnikova et al. 2015).

Nitrate-mediated cytokinin induction results in an increase in the non-hydroxylated cytokinins [referred to subsequently as isopentenyladenine (iP)-type cytokinins] in the root, and subsequent conversion predominantly in the root and stem to hydroxylated forms [referred to subsequently as *trans*-zeatin (*tZ*)-types] (Sakakibara 2006). The current model proposes that, following N induction of cytokinin biosynthesis, iP-type cytokinins are converted to *tZ*-type cytokinins which are then translocated from root to shoot via the xylem to regulate shoot growth. In contrast, iP-type cytokinins are generally found to be the major cytokinin forms in the phloem of *Arabidopsis*, indicating a potential role of iP-type cytokinins in signaling from shoot to root (Hirose et al. 2008). In a split-root experiment coupled to transcriptome analysis, Ruffel et al. (2011) showed that cytokinins play a key role in a shoot-to-root systemic signaling of N that controls root morphology. They suggested that cytokinins are crucial components of a local NO_3^- signal, and of a systemic root-shoot-root signal integrating the NO_3^- status of the plant (Ruffel et al. 2011).

Following recognition of the accumulating cytokinin free-bases by their receptors (Lomin et al. 2015), a downstream signaling cascade is initiated resulting in expression of response regulators (*RR*s) (Hwang and Sheen 2001). The type-B *RR*s serve as direct transcriptional regulators for certain target genes, including type-A *RR*s. A negative feedback regulation exists between type-B and type-A *RR*s. Upregulation of type-A *RR*s by NO_3^- was shown in both maize (Sakakibara et al. 1998) and *Arabidopsis* (Taniguchi et al. 1998). Recently, Shtratnikova et al. (2015) showed that NO_3^- and not NH_4^+ increased the expression of *Arabidopsis* type-A *RR5::GUS* reporter constructs.

Up to now, the molecular mechanisms and regulation of NO_3^- assimilation and its interaction with C metabolism have been studied mostly in *Arabidopsis*, and continued efforts to further understand these processes are necessary to achieve an improvement of NUE in perennial ryegrass pasture systems. The aim of this work was to develop a spatiotemporal understanding of the events taking place during early NO_3^- assimilation in perennial ryegrass, i.e. between NO_3^- supply and subsequent N-associated growth response of young plants grown previously under steady-state low N availability. Of particular interest was the interaction between N and C metabolism in different tissues, and the putative involvement of cytokinin in perennial ryegrass. We analyzed the physiological response, metabolic profile and gene expression of the roots and the shoots of 10-week-old perennial ryegrass plants grown on unfertilized soil and subsequently exposed to high levels (5 m*M*) of KNO_3 for up to 7 days. The metabolic analysis included a profiling of the WSCs across their DP, and a profiling of the amino acids. Cytokinin content was measured in response to 5 m*M* KNO_3 or KCl treatment of steady-state N-deficient perennial ryegrass plants, and the expression of *LpRR* genes was monitored by quantitative reverse transcription polymerase chain reaction (qRT-PCR), with the expectation that perennial ryegrass possesses a cytokinin-mediated N-signaling/relay system similar to that characterized in model plant species.

Materials and methods

Plant material

Lolium perenne cv. Grasslands Nui seedlings were grown for 10 weeks in a pot experiment conducted at the University of Canterbury glasshouses (43°31′48″S; 172°37′13″E) using unfertilized bark-free soil commercially acquired near Christchurch, New Zealand (Table S1, Supporting information). Single perennial ryegrass seeds were sown directly onto perlite filling a 1.5-ml Eppendorf tube, the bottom of which had previously been cut off. The tubes with the single seed-derived plant were placed on individual pots until plant establishment. Soil water content was maintained by automatic watering for 5 min daily by mist emitters located throughout the room. Roof ventilation was set to maintain 22°C temperature. Once established, plants were removed from their pots, the roots were washed and their tubes were placed into fitted holes within polyvinyl chloride channels in a homemade hydroponic system. Seedlings

were maintained for 1 week in Hoagland-N liquid medium (N-free Hoagland medium, BioWorld, Dublin, Ohio, USA) for acclimation to liquid growth conditions.

Treatments with 5 m*M* KCl or KNO_3 defined day zero (d0) of the experiment, were performed by direct addition into the liquid medium after a week-long acclimation period. Temperature, pH and electrical conductivity of the medium were monitored daily and, when required, the water level and pH were adjusted. Perennial ryegrass roots and shoots were destructively harvested on days one (d1), three (d3) and seven (d7) and the plant material was immediately flash frozen in liquid nitrogen and subsequently stored at −80°C. Tissue samples harvested from five plants were pooled and subsequently treated as one biological replicate. Total N and total C content of roots and shoots were quantified by Isotope Ratio Mass Spectrometer at the Department of Soil and Physical Sciences, Lincoln University, Christchurch, New Zealand. Three biological replicates were analyzed.

Metabolite analysis

WSCs of low (LMW)- and high-molecular weight (HMW) were extracted and subjected to LC-MS analysis as described in Harrison et al. (2009). Oligosaccharides of DP from DP2 to DP35 were quantified based on the extracted ion chromatogram using methods largely described in Cao et al. (2013). A group of adduct ions that represent the same DP of oligosaccharides were quantified, summed up and statistically analyzed (see Appendix S1 for the detailed procedures). Amino acids, organic acids and phenylpropanoids were extracted, derivatized and determined by using GC-MS as described in Cao et al. (2013). The GC-MS data were processed using the publicly available R package TARGETSEARCH (Cuadros-Inostroza et al. 2009). Compounds detected and quantified were: citric acid, malic acid, fumaric acid, Ala, Asn, Asp, Glu, Gln, Gly, Ile, Leu, Lys, Orn, Phe, Ser, Thr, Trp, Tyr and Val. Perennial ryegrass samples were extracted as follows: powdered, freeze-dried plant material (25 mg) was weighed into 2-ml screw-cap tubes and 250 µl of chloroform ($CHCl_3$) added. Samples were briefly vortexed and 900 µl of methanol, 250 µl of ultrapure (MilliQ) water, 50 µl of ribitol (0.2 mg ml^{-1} water) and 50 µl of pentadecanoic acid (2 mg ml^{-1} $CHCl_3$) were added. Samples were vortexed again and mixed by shaking for 15 min at 60°C. Extracts were centrifuged for 5 min at 13 000 *g* and the two phases were transferred into separate tubes. Organic acids and amino acids were determined in the aqueous phase. Polar extracts were dried in a vacuum centrifuge (Savant) for 60 min in a 2-ml GC sample vial. About 50 µl of 20 mg ml^{-1} solution of methoxyamine hydrochloride in pyridine was added and incubated at 30°C for 90 min with occasional shaking after mixing. About 80 µl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide reagent (Sigma-Aldrich, Auckland, New Zealand) was added, samples incubated at 37°C for 30 min and finally at room temperature for 120 min with occasional shaking. Samples were transferred to 250-µl glass inserts and 1 µl was injected into the Shimadzu QP-2010 GC-MS (Shimadzu Corporation, Kyoto, Japan) with a 40:1 split ratio. Helium was used as the carrier gas with the column flow rate of 1 ml min^{-1}. The oven was held at 70°C for 5 min and then heated at 5°C min^{-1} to 310°C and held at that temperature for 1 min. The compounds were resolved on a Zebron ZB-50 column (Phenomenex (Torrance, CA, USA), 30 m × 0.25 mm I.D. × 0.25 µm film thickness). The mass spectrometer was operated in scan mode monitoring the mass range 50–600 m z^{-1} every 0.5 s. Three biological replicates, each comprising five plants, were analyzed.

Cytokinin measurements

Prepared biological triplicates were extracted and purified using the method published previously by Dobrev and Kamínek (2002) with some minor modifications (Antoniadi et al. 2015). Samples, 3–5.5 mg dry weight (DW), were extracted in 1 ml of modified Bieleski buffer (60% MeOH, 10% HCOOH and 30% H_2O) together with a cocktail of 18 stable isotope-labeled cytokinin internal standards (0.2 pmol of cytokinin bases, ribosides, *N*-glucosides, 0.5 pmol of *O*-glucosides and nucleotides) to check recovery during purification and to validate the determination. The samples were purified using a combination of C_{18} (100 mg ml^{-1}) and mixed mode-cation exchange (MCX) cartridges (30 mg ml^{-1}). The eluates were evaporated to dryness and dissolved in 20 µl of the mobile phase used for quantitative analysis. The samples were analyzed by the LC-MS/MS system consisting of an ACQUITY UPLC® System (Waters, Massachusetts, USA) and Xevo® TQ-S (Waters, Massachusetts, USA) triple quadrupole mass spectrometer. Quantification was obtained using a multiple reaction monitoring mode of selected precursor ions and the appropriate production (Svačinová et al. 2012).

Gene expression

RNA isolation and cDNA synthesis

Total RNA was extracted from up to 100 mg of frozen root and shoot samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's

instructions and immediately stored at −20°C. Genomic DNA contamination was prevented by using an RNase-Free DNase Set (Qiagen). The concentration and purity of the total RNA was assessed using a Nanodrop™ spectrophotometer, and by electrophoresis on 1% (w/v) agarose gel. Approximately 1 µg of total RNA was converted to cDNA through RT in a 20 µl reaction with 50 U of Expand Reverse Transcriptase (Roche, Mannheim, Germany), 50 pmol of oligo (dT) primers and 100 pmol of random hexamer (pdN6) primers. The final reaction mix was incubated at room temperature for 10 min, then 42°C for 60 min and then at 70°C for 15 min to deactivate the enzyme. The cDNA was diluted 10-fold with nanopure water and stored at −20°C.

Target gene isolation and sequence analysis

Sequences of candidate *RR* gene family members in perennial ryegrass were determined through BLAST searching the NCBI database and a RNA-Seq transcriptome database containing 169 862 assembled sequence contigs of 595 bp in average length generated using an Illumina HiSeq2000 genome analyzer (Macrogen Ltd, Seoul, Korea). Perennial ryegrass cv. Nui was used for transcriptome data generation. A pool of combined RNA samples extracted from multiple developmental stages of leaves, flower spikes and seeds was used to construct the cDNA library. All available sequences of *RR* gene families in the GenBank database (Table S3) in perennial ryegrass and closely related species including *Brachypodium distachyon*, *Oryza sativa*, *Oryza brachyantha*, *Zea mays*, *Triticum aestivum*, *Festuca pratensis*, *Hordeum vulgare*, *Setaria italica* and *A. thaliana* were used as query sequences to BLAST search the perennial ryegrass transcriptome database using PRFECTBLAST 2.0 software. The putative sequences were verified via BLAST searching the GenBank database and via multiple sequence alignment with representative ortholog sequences in closely related species.

Neighbor-joining phylogenetic trees of the newly identified sequences and their orthologs (listed above) were created using CLUSTALX2 software with 1000 bootstrap replicates. The phylogenetic tree was visualized with TREEVIEW X software. The tree was rooted with an out-group sequence from *L. perenne* (Fig. S1). The GenBank accession numbers for the nucleotide sequences are listed in Table S3.

Quantitative reverse transcription polymerase chain reaction

qRT-PCR was used to measure the relative gene expression of the individual family members. Specific PCR primers were designed for each *LpRR* gene family member using PRIMER PREMIER 6.20 (Table S4). In most cases, four primer pairs were designed and the best pair was chosen for gene expression analysis after PCR testing. A volume of 20 µl was used for all qRT-PCRs in a Rotor-Gene Q system real-time PCR instrument (Qiagen), using a homemade SYBR Green-based master mix. PCR products for each target sequence were Sanger sequenced to confirm homology to genes already identified in various gene databases (e.g. NCBI). qRT-PCR systems were then optimized. Two reference genes, *ELONGATION FACTOR 1 ALPHA* (*EF*) and *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*), were used as internal controls. For each cDNA sample, the Ct values of each target gene were corrected using a correction factor calculated as described previously (Song et al. 2012). Three technical replicates were carried out for one sample set of five pooled plants. The expression values relative to *EF* and *GAPDH* were calculated based on the methods of Pfaffl (2001) and modified as described in Song et al. (2012) (Table S5).

Statistical analysis

The variables were subject to a two-way ANOVA with tissue (root and shoot) and time (0 = pretreatment, and 1, 3, 7 for 1, 3 and 7 days after N addition, respectively) as the factors. Significant effects were defined by Dunnett's multiple comparisons test. Results of an ANOVA were considered statistically significant when $P < 0.05$. Relative abundance in C units in DP2, DP3–6 and HMW WSCs and in amino acids was considered significantly different relative to pretreatment (d0). Similarly, the statistical significance in the cytokinin contents in the KCl- or KNO_3-treated plants at d1 and d7 was estimated relative to d0.

Results

Physiological and growth responses to added nitrogen

To investigate the early response of KNO_3 addition on N-deficient plants, total C and N contents were measured in plants grown initially in unfertilized soil and then exposed to 5 m*M* KNO_3 or KCl (control) for 1, 3 or 7 days. Shoot-N content was higher (Fig. 1A) and C:N ratio was lower than in the root (Fig. 1B). The N content in N-treated roots increased rapidly within d1 of N treatment, and was associated with a decrease in the C:N ratio, whereas the shoot dynamics changed more gradually over the course of the experiment. To determine the NUE of perennial ryegrass plants in

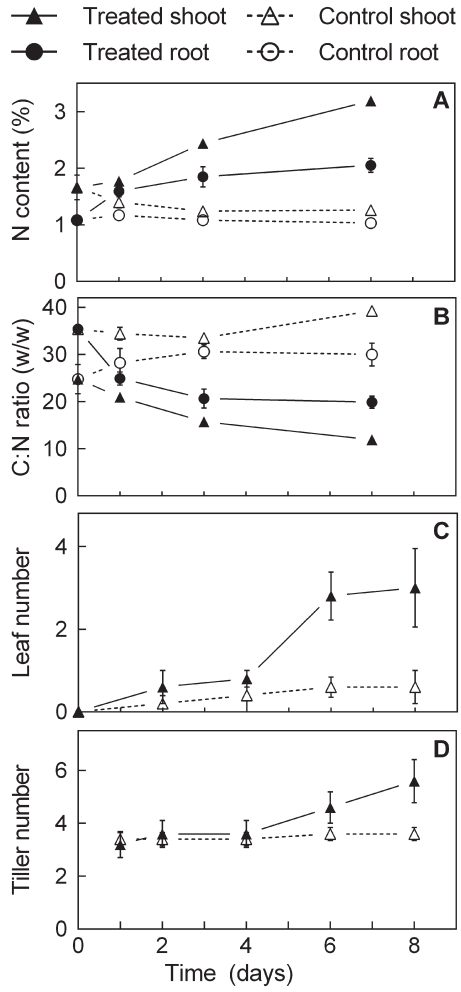

Fig. 1. Effect of NO_3^- addition on total N content (A), C:N ratio (B), leaf appearance (C) and tiller number (D) of perennial ryegrass plants. Plants were treated with 5 m*M* KCl (control) or 5 m*M* KNO_3 (treated) on day zero. Data are means ± SE, n = 4 pools of five plants for N content and C:N ratio; n = 5 for leaf number and tiller number.

hydroponics, leaf and tiller numbers were measured and had increased significantly within 6 days following KNO_3 addition (Fig. 1C, D).

Water-soluble carbohydrates

To understand the dynamics of metabolic changes over time in response to N addition, a metabolic profiling was conducted. N-deficient plants at d0 had accumulated WSCs in above- and below-ground tissues with relatively greater storage abundance of stored C in the shoot (Fig. 2A, B). When calculated per unit of C, disaccharides were the most abundant WSCs regardless of the tissue type. The WSCs with DP from three to six, referred to here as LMW WSCs, were less abundant relative to the disaccharides. By contrast, the relative contribution in terms of C units from the HMW WSCs increased from DP7 to DP14 and then decreased for the WSCs of higher DP. Shoot and root tissues presented different profiles in the relative C contribution from the WSCs. Before N addition, C units present in DP2 had accumulated slightly more in the shoot (Fig. 2C). There were no statistically significant differences in the abundance of other LMW WSCs (DP3–6) between tissue types (Fig. 2D). However, more substantial differences were observed between root and shoot for the HMW WSCs (Fig. 2E). As a general trend, the higher the DP, the more likely it was to be accumulated in the shoot rather than in the root (Fig. 2F). HMW WSCs were three times more prevalent in the shoot than in the root at pretreatment (d0). In particular, DP31 was the most prevalent WSC in the shoot and provided seven times more C than did DP31 in the root.

A decrease in all WSCs was observed in response to N addition (Fig. 2). However, the dynamics of responses differed between LMW and HMW WSCs, and tissue types. In the root, the breakdown of HMW WSCs was observed mainly between d1 and d3 and subsequently stabilized (Fig. 2B), whereas in the shoot the total HMW WSCs decreased gradually after KNO_3 addition (Fig. 2E). Although LMW WSCs in the root were significantly reduced by d1, changes in the relative abundance in C units of LMW WSCs in the shoot had become significantly reduced only by d7.

Metabolites associated with glycolysis, TCA cycle and amino acids

Figure 3 shows the variation in relative abundance (peak intensity) of amino acids and metabolic intermediates of the tricarboxylic acid (TCA) cycle immediately before and at 1, 3 and 7 days following 5 m*M* KNO_3 treatment of perennial ryegrass plants in hydroponic solution. The TCA cycle intermediates, citric acid, fumaric acid and malic acid, decreased significantly and rapidly within a day after N supply in the root (Fig. 3E) but fumaric acid and malic acid did not change significantly over time in the shoot. The major N-carrier amino acids Gln, Asp and Asn accumulated after N treatment with similar patterns between tissues and showed comparatively higher relative abundance in the shoot than in the root (Fig. 3D, F). Glutamine accumulated within the first day, and subsequently decreased in the shoot. The increase in Asp and Gln represented the strongest difference in relative abundance (difference of peak intensity) observed after KNO_3 addition. Aspartic acid and Asn were characterized by a notably longer accumulation, reaching their highest intensity by d3, followed by a plateau (Fig. 3D).

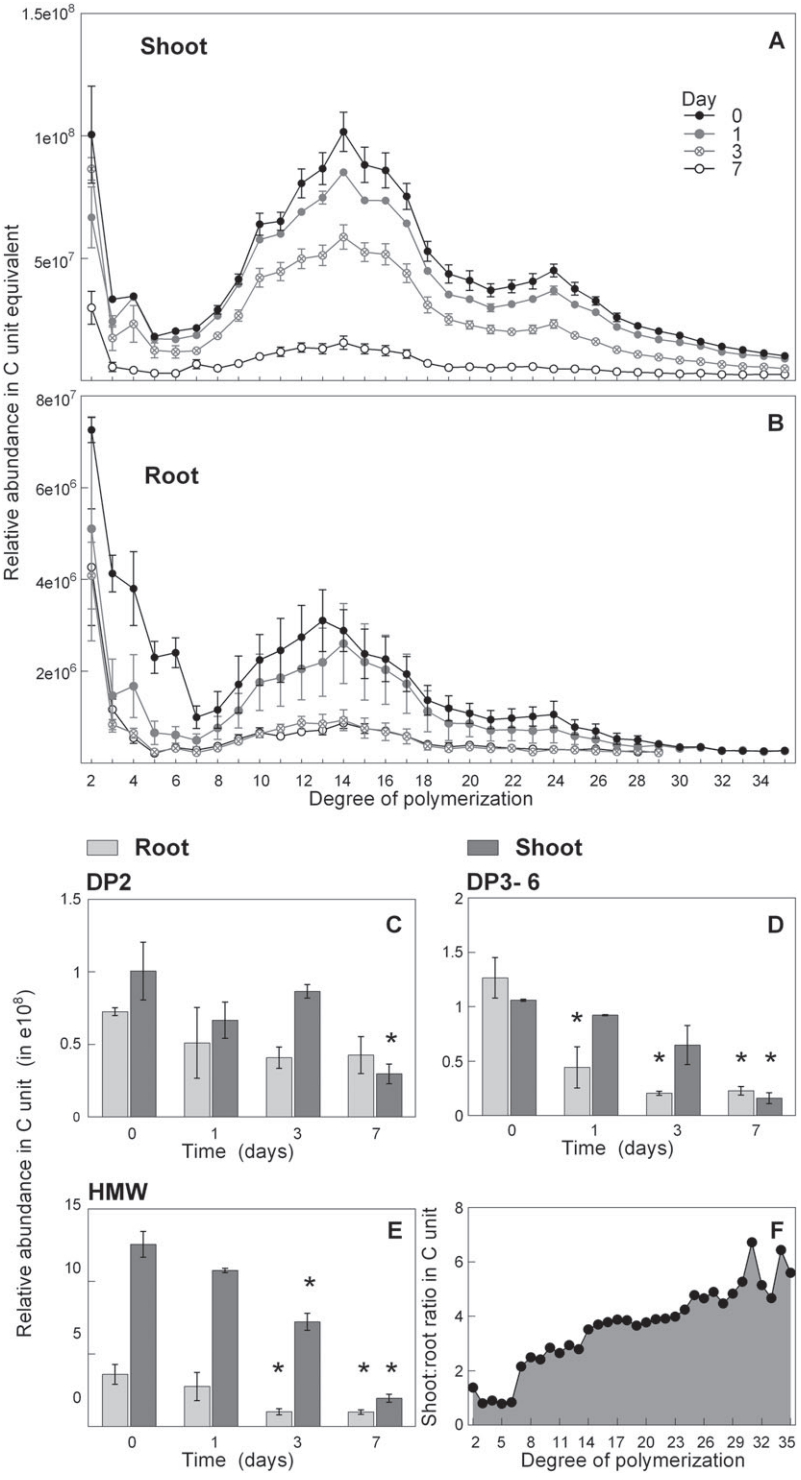

Fig. 2. Relative abundance in C units of WSCs measured over the 7-day period following 5 m*M* KNO_3 addition to the hydroponic solution of N-deficient perennial ryegrass plants. Relative abundance in C units was calculated by multiplying peak intensity by DP. WSC profiling in shoots (A) and roots (B); relative abundance of WSC of DP two (C), three to six (D) and HMW (E) and ratio of shoot to root relative abundance in fructans at d0 (F). 0 = pretreatment, and 1, 3 and 7 for 1, 3 and 7 days after N addition respectively. Data are means ± SE, n = 3, where each replicate is a pool of five plants. Asterisks indicate a significant difference from the pretreatment (0) in root and shoot tissue.

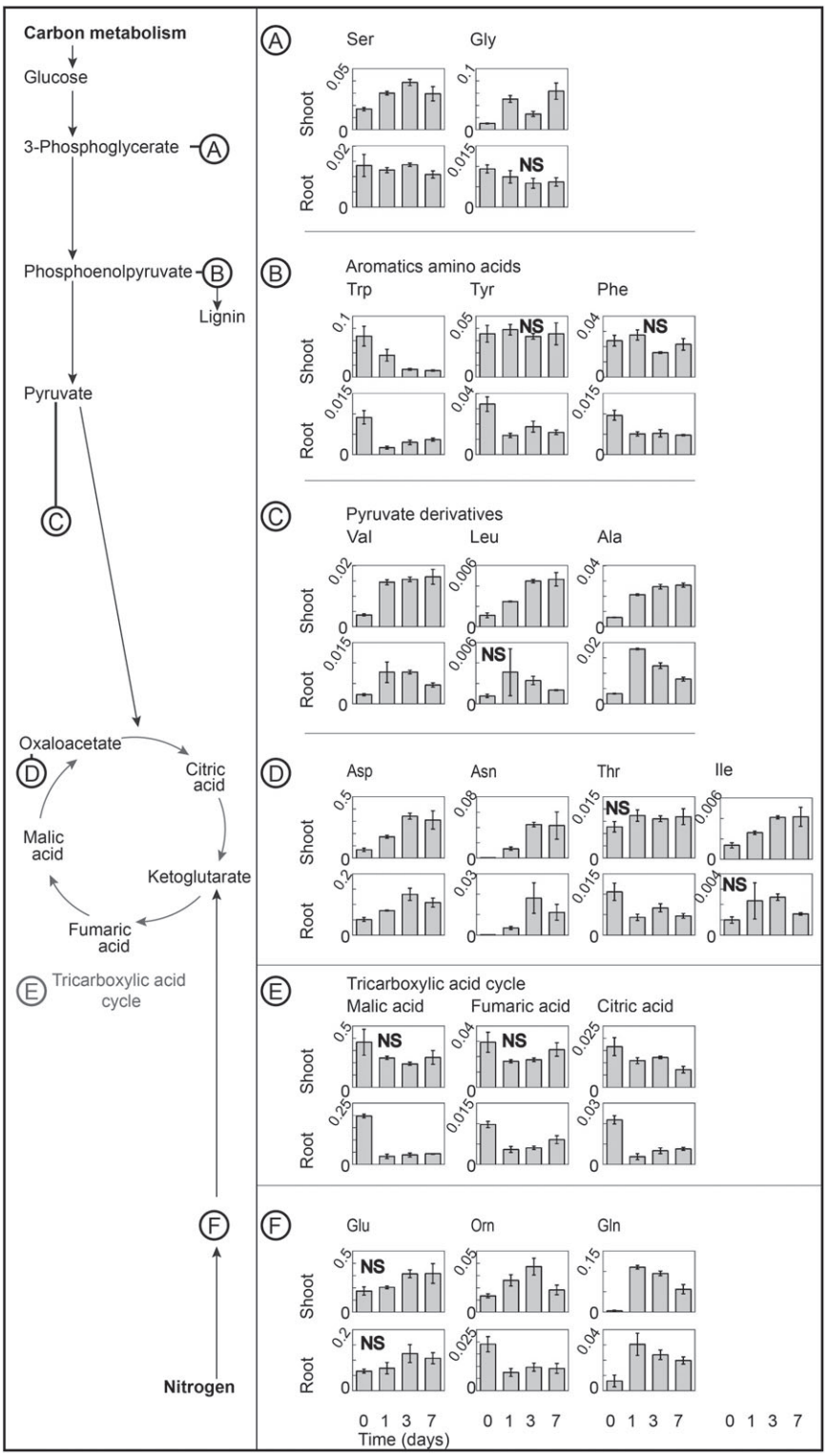

Fig. 3. Mapping of metabolite changes in values representing relative abundance alongside known pathways immediately before and at 1, 3 and 7 days following 5 m*M* KNO_3 treatment of perennial ryegrass plants in hydroponic solution. 0 = pretreatment, and 1, 3 and 7 for 1, 3 and 7 days after N addition, respectively. NS: not statistically significant compared with pretreatment within the same tissue; n = 3 where each replicate is a pool of five plants.

This pattern was also observed for Ile, Leu and Ala in the shoot (Fig. 3D, C), and was distinct from their patterns in the root: root Ile and Leu did not change significantly, and root Ala accumulated during d1 and subsequently decreased. More complex variations were recorded for Gly, Thr and Val, for which the trends contrasted over time and between tissues (Fig. 3A, D, C). Among the other N-rich amino acids, Glu did not change significantly over the course of the experiment, and His and Arg trends remained unknown due to a lack of annotations (Fig. 3F). A rapid decrease in the aromatic amino acids Trp, and root Tyr and Phe, was observed following N addition (Fig. 3B).

Cytokinin content and expression of cytokinin *RR* genes

As a general trend, N addition resulted in a significant augmentation of the cytokinin content relative to d0 (Fig. 4; see Table S2 for the complete dataset). In particular, the *t*Z-types were characterized by a highly significant peak at d1. Along with an increase in *t*Z ribosyl monophosphate (*t*ZRMP), iPRMP accumulated in response to N, although the response of iPRMP appeared similar to that in both the KNO_3 and KCl treatment of the root at d1. An accumulation of *t*Z riboside (*t*ZR) and iPR occurred following N addition in both tissues. The iPR increase was also associated with a response to KCl treatment in the shoot.

The N induction of the active free-bases was stronger in the shoot compared with the root. Indeed, the quantity of *t*Z and iP was approximately twice as great in the shoot as in the root following N treatment (Fig. 4A). During the course of the experiment, *t*Z and iP levels remained significantly higher relative to pretreatment up to d7 in the shoot, but only up to d1 in the root. *t*Z accumulation was specific to N treatment (Fig. 4A). In contrast, accumulation of iP-types was also observed in response to KCl (Fig. 4). *t*Z glucosides increased significantly in response to N treatment: by d1 for *t*Z *O*-glucoside (*t*ZOG) in the shoot; by d7 for *t*ZOG, *t*ZROG, *t*Z-7-glucoside (*t*Z7G) and *t*Z-9-glucoside (*t*Z9G) in the shoot and *t*ZROG and *t*Z9G in the roots (Fig. 4A). iP9G increased in response to KCl at d1 in the roots (Fig. 4B). The dihydrozeatin-type cytokinins were all below 3 pmol g DW^{-1} in perennial ryegrass, regardless of the treatment type, tissue type and time point (Fig. 4, Table S2).

In contrast to the *trans*-cytokinins and iP-type cytokinins, none of the *cis*-forms increased following N treatment. The majority of the changes in the *cis*-forms occurred following the KCl treatment (Fig. 4B). In particular, *c*Z riboside (*c*ZR) and *cis*-zeatin (*c*Z) levels increased in the shoots, with a smaller increase of *c*ZR at d1 in the roots. The *cis*-glucosides, *c*Z *O*-glucoside (*c*ZOG) and *c*ZROG, were detected at the greatest level of all the cytokinins at d0, noting that *c*ZOG was detected at its greatest level in the shoot tissue, whereas *c*ZROG was at its greatest level in the roots. KCl treatment induced a significant decrease in *c*ZROG at d7 in the shoot, and a similar but not significant decrease in *c*ZOG. In addition, decreases in *c*ZRMP were detected following both KCl and KNO_3 treatments.

To decipher whether the variation in cytokinin activated a cytokinin signaling pathway, the expression of putative cytokinin *RR*s was recorded using qRT-PCR analysis for relative quantification (Fig. 5). All of the upregulated *LpRR* family members were type-A *LpRRs*. However, *LpRR3* was downregulated in both shoots and roots. All type-B *LpRRs* were downregulated following KNO_3 addition. The only *LpRR* family member remaining within twofold changes before and after KNO_3 treatment, in both shoot and root tissues, was type-A *LpRR6*. Overall, the root and shoot profiles contrasted mainly by the responses of the various *LpRR* family members and their associated timing of expression. In the root, four of the eight *LpRR* members identified remained within twofold changes following N addition, whereas a shoot-specific downregulation was identified for *LpRR2, LpRR12a* and *LpRR12b*. Therefore, most of the *LpRRs* were downregulated in the shoot, especially at d1, which contrasted with fewer *LpRR* members regulated in the root.

Discussion

HMW WSCs supply carbon for nitrogen metabolism

Consistent with Louahlia et al. (2008), N-deficient perennial ryegrass plants accumulated WSCs, and showed a limited assimilation of NO_3^-, with reduced levels of some free amino acids particularly at d0 for the nitrogenous amino acids Gln and Asn, and the aliphatic amino acids Leu, Ile, Val and Ala (Fig. 3). When N was resupplied, the opposite trends were recorded (Figs 2 and 3).

The total N content was greater in shoot than in root tissue, irrespective of the N treatment (Fig. 1A), indicating that the shoots of perennial ryegrass are the primary organ for N storage and utilization. Additionally, as most of the HMW WSCs were located in the shoots rather than in the roots (Fig. 2A, B, F), this suggests that the shoots are also the main organ for C storage in young perennial ryegrass plants. Under our experimental conditions, N treatment was associated with a shift of amino acids toward a greater accumulation in the shoot rather than in the root (Fig. 3), suggesting that the amino acids

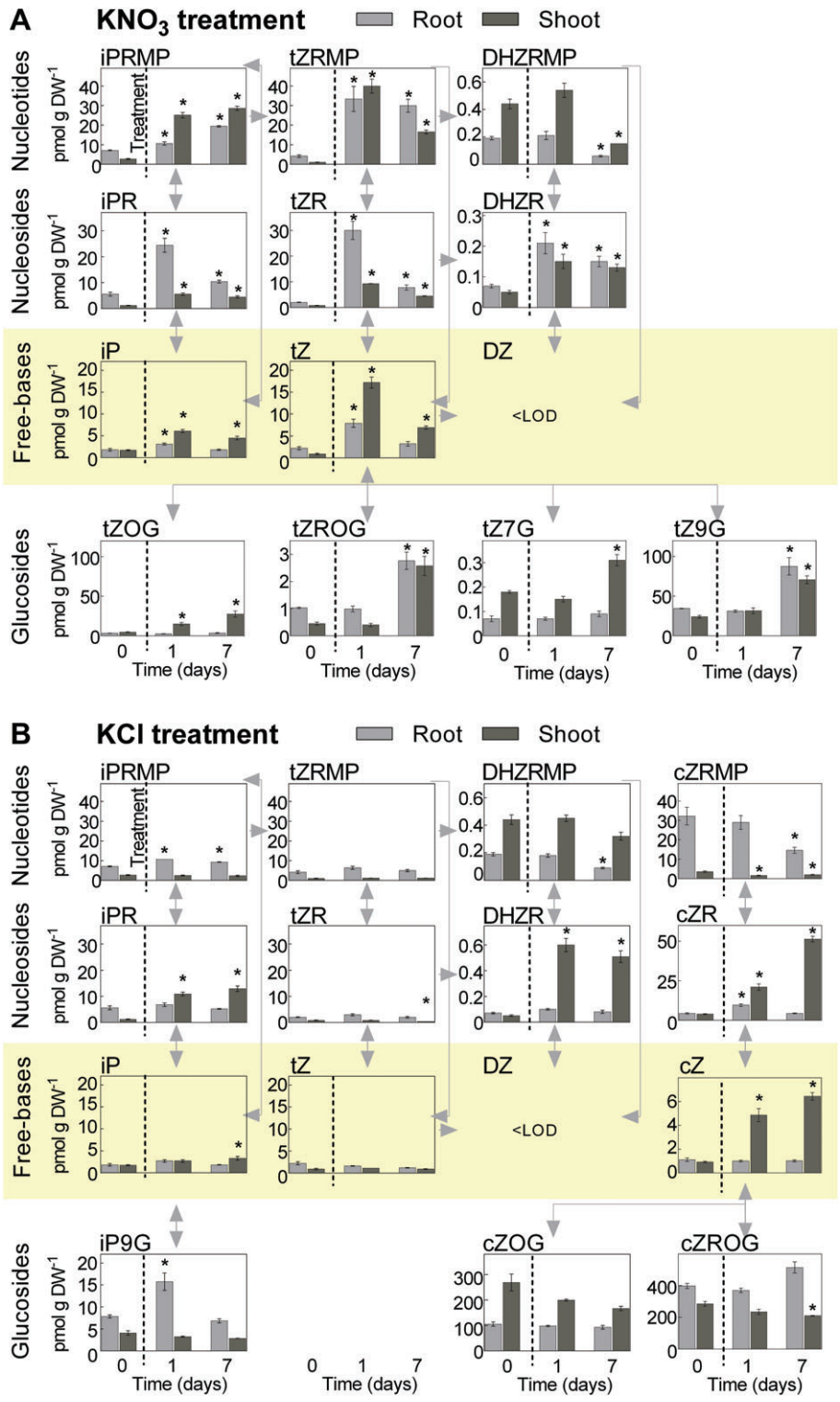

Fig. 4. Cytokinin content of perennial ryegrass roots (light gray) and shoots (dark gray). 0 = pretreatment, and 1, 7 for, respectively, 1 and 7 days after 5 m*M* of KNO_3 treatment (A) or KCl treatment (B). Data are means ± SE, n = 3 where each replicate is a pool of five plants. Dihydrozeatin (DZ) content was below 1 pmol g DW^{-1} and other non-significantly changing cytokinin forms are not shown. The complete dataset can be found in Table S2.

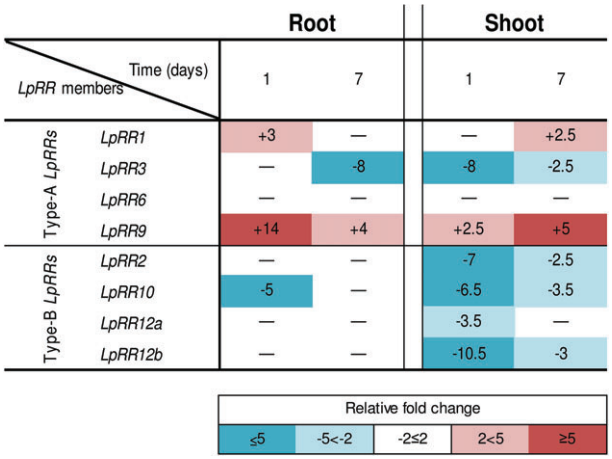

Fig. 5. Relative expression of cytokinin *RR* gene family members in root and shoot tissues of perennial ryegrass. Positive (+) or negative (–) values were fold changes relative to the expression level immediately before treatment, calculated using reference genes *GAPDH* and *EF* as internal controls. Numbers indicated in each cell correspond to relative fold change in expression. 0 = pretreatment, and 1 and 7 for 1 and 7 days after N addition, respectively. Each value represents the mean of three technical replicates using a cDNA from a pool of five plants as the qRT-PCR template.

could be synthesized or accumulated preferentially in the above-ground parts of perennial ryegrass to support shoot growth in response to N addition. Our results are consistent with the study of Bowman and Paul (1988) who showed, following a NO_3^- treatment on N-limited perennial ryegrass plants, that an initial phase of NO_3^- reduction in the root during the first 12 h following NO_3^- treatment was followed in time by a greater NO_3^- reduction in the shoot (Bowman and Paul 1988).

Three main phases of responses were identified following NO_3^- treatment to N-depleted perennial ryegrass plants. The first phase took place within 1 day and highlighted the importance of the root in the initial N response (Fig. 1A). It was characterized by a rapid remobilization of C from root LMW WSCs (Fig. 2B, D), combined with a significant increase in root N content and a rapid biosynthesis of free amino acids (Fig. 3). Initially, the HMW WSCs may have been maintaining the pool of LMW WSCs, especially in the shoot. The second phase occurred between d1 and d3. The shoot became the main tissue for N accumulation (Fig. 1A) and the main source of C from WSCs by remobilization of a large pool of HMW WSCs that had accumulated in the shoot under N-limiting conditions (Fig. 2A, E). In contrast, root N content had stabilized by d3 (Fig. 1A) and the root WSCs reserve had almost become exhausted (Fig. 2B, D). Most amino acids stabilized by d3, with a higher abundance noted in the shoot (Fig. 3). The third phase was in place by d7. By that time, the shoot N content had continued to increase (Fig. 1A). Interestingly, the shoot HMW WSCs continued to steadily breakdown, whereas the root reserves were exhausted (Fig. 2B, E). Some of the amino acids that initially increased in response to N addition in the root decreased at d7 (Fig. 3), suggesting that the peak of amino acid production was over by this time. Our data indicate a decline in NO_3^- assimilation within 1 week of N fertilization in the root, and to a lesser extent in the shoot.

Interestingly, this trend was only observed for the amino acids Gln and Orn in the shoot. Nitrate assimilation into amino acids is initiated by the reduction of NO_3^- to nitrite by the enzyme nitrate reductase. Nitrite is subsequently converted to NH_4^+ by the plastidic or chloroplastic nitrite reductase enzyme. Glutamine and Glu are considered to be the primary products of NH_4^+ assimilation with the GS/GOGAT enzymes (glutamine synthetase and glutamate synthase) acting as catalysts in a cycle in which Glu is both a substrate and end product.

Considering that Gln is a precursor to all other amino acids, the stable relative abundance of all the amino acids, with the exception of Gln and Orn, in the shoot by d7 might be explained by either a steady rate of biosynthesis that could potentially affect Gln mobilization or an amino acid import from the root to the shoot or, possibly, a lower depletion of amino acids in the shoot by d7.

Notably, shoot DP2 had only decreased significantly by d7 (Fig. 2C). However, caution must be taken when estimating the C fluxes from measurements of steady-state level of DP2, because of issues of stability and compartmentalization that can lead to misleading biological interpretation (Fernie et al. 2005).

TCA cycle intermediates decrease in response to nitrogen

Treatment with KNO_3 was associated with a decrease in the roots of the TCA cycle intermediates citric acid, fumaric acid and malic acid (Fig. 3E). This is opposite to the results from a metabolomic analysis of mature perennial ryegrass plants (Rasmussen et al. 2008). They showed that malate, succinate and citrate were all higher for plants grown at steady-state high N supply compared with plants with reduced N supply. This might represent a developmental response, as our results are consistent with increased levels, especially in the roots, of citric acid, fumaric acid and malic acid recorded in *Arabidopsis* plants that were N starved for 10 days (Krapp et al. 2011). Previously, Tschoep et al. (2009) reported that *Arabidopsis* plants under a mild but sustained N limitation showed increasing fumaric acid content. In addition, *Arabidopsis* leaves have often been reported to have very high levels of fumaric acid, sometimes exceeding those

of starch and soluble sugars (Chia et al. 2000). Fumaric acid has thus been hypothesized to act as a temporary C sink for photosynthate which can be remobilized to yield energy and C (Chia et al. 2000), and may have a function in C partitioning between different plant parts (Tschoep et al. 2009). *Arabidopsis* knockout mutants of a cytosolic fumarase suggested an important role of the fumaric acid/malic acid homeostasis in maintaining C metabolism, as well as rapid N assimilation and growth on high N (Pracharoenwattana et al. 2010). Under our experimental conditions, fumaric acid and malic acid decreased statistically significantly in the root and did not decrease significantly in the shoot of perennial ryegrass in response to KNO_3 treatment, which suggests that fumaric acid and, potentially, malic acid could act as flexible alternative C store influenced by the N status in a tissue-specific manner.

Cytokinins respond to both the presence and absence of nitrogen

Plants possess intricate regulatory machinery able to coordinate N use with C metabolism, and thus the control of the C/N ratio (Nunes-Nesi et al. 2010). Here, we have shown that changes in cytokinin occurred in response to the addition of both KNO_3 and KCl (Fig. 4). According to Takei et al. (2004), changes in cytokinin levels occurred within 24 h following the resupply of N to N-starved maize plants. In agreement with Takei et al. (2004), our data show extensive accumulation and also metabolism of cytokinin, both in roots and shoots, at 24 h following N supply. Even within 24 h, *t*ZOG had increased in the shoots and, over 7 days, both storage (*t*ZOG, *t*ZROG and *c*ZROG) and inactivated forms (*t*Z7G and *t*Z9G) had accumulated, indicating that cytokinin homeostatic mechanisms were activated rapidly in shoots.

Two maize type-A cytokinin *RRs*, that were upregulated following addition of NO_3^-, were initially described as N-responsive genes but were subsequently shown to be activated directly by cytokinin and cytokinin-mediated N responses and not by N itself (Takei et al. 2001). We have shown that, following N addition to perennial ryegrass, the upregulated *LpRRs* were all type-A *LpRRs* (Fig. 5). However, we also showed that, within 24 h of N addition, all the type-B *LpRRs* in the shoot were downregulated, indicative of a feedback mechanism dampening the NO_3^--induced cytokinin responses. Clearly, cytokinin biosynthesis, metabolism and signaling are associated with the transition of perennial ryegrass from N-starved to N-replete.

Changes in cytokinin content were observed in response to KCl, particularly the *c*Z- and the iP-type cytokinins (Fig. 4B). As found in other members of the Poaceae (Gajdošová et al. 2011), we detected relatively high levels of *c*Z-type cytokinins in perennial ryegrass. In particular, the storage forms *c*ZOG and *c*ZROG were detected at one order of magnitude greater than any other cytokinin at d0, at which time the plants had been growing on unfertilized soil for 10 weeks. The *c*Z- and iP-type cytokinins are normal constituents of certain tRNAs (Sakakibara 2006). Schäfer et al. (2015) suggested that various stress conditions induce tRNA turnover pathways. They noted that *c*Z-containing tRNA can be formed from hydroxylation of the iP-containing tRNA, and the action of an unknown enzyme on these tRNAs could release *c*Z/*c*ZR forms and iPR forms (Schäfer et al. 2015). Consequently, enhanced tRNA turnover could explain the increase in both *c*Z- and iP-type cytokinins in response to KCl treatment in our experiment. Interestingly, wheat plants exposed to ammonia as sole N source also showed enhanced levels of both *c*Z- and iP-type cytokinins (Garnica et al. 2010). Our data are consistent with the hypothesis presented by Gajdošová et al. (2011) that *c*Z and/or its derivatives might maintain minimal levels of cytokinin under growth-limiting conditions necessary for plant survival and subsequent recovery (Gajdošová et al. 2011).

In conclusion, our results provide a spatiotemporal characterization of amino acids, TCA intermediates and WSCs, as well as cytokinin biosynthesis, metabolism and signaling of N-limited young perennial ryegrass plants following KNO_3 treatment. We identified an initial remobilization of LMW WSCs within a day of N treatment, followed in time by a remobilization of a larger pool of HMW WSCs in the shoot. This was associated with a greater accumulation of N and amino acids in the shoot. Variations in the cytokinin content and *LpRR* gene expression suggest long-distance root/shoot signaling within a day of KNO_3 treatment, and ongoing input from cytokinin over the 7 days. Our spatiotemporal profiling represents a translational step that builds on fundamental research in model plant systems and aims to thoroughly describe NO_3^- assimilation and some key responses in an agriculturally important pasture species, *L. perenne*. We reveal some intricate root/shoot dynamics of WSC remobilization, N assimilation and subsequent growth responses to KNO_3 treatment.

Author contributions

M. H. T., P. E. J. and J. L. conceived this study. J. R. and J. L. designed, carried out the experiments and analyzed the data. J. S. analyzed the transcriptome; Q. G. assisted with experiments; M. C., K. F., J. H. and C. J. performed the metabolomics and O. N. provided the cytokinin

analyses. J. R. wrote the manuscript with significant input from P. E. J. and comments from all other authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Procedures for oligosaccharide quantification.

Table S1. Physical and chemical properties of the soil.

Table S2. Full cytokinin profiling.

Table S3. GenBank accession numbers.

Table S4. *LpRR* primer sequences.

Table S5. *LpRR* gene expression profiles in response to KNO_3 treatment.

Fig. S1. Phylogeny of *LpRR* gene family.

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